ERRATA, VOL. XX.

p. 640, Fig. 1, Method 1, 8 c.c. : XXVI *should read* XXVII
„ „ Method 2, 5 c.c. : XXVI *should read* XXVII

p. 666, the foot-notes *should read:*

* Calculation from the results of Medes gives 122 at 7·5° and 182 at 21°.
† „ „ „ „ 34·5 at 7·5° and 61 at 21°.

CXII. STUDIES ON THE PHYSIOLOGY OF PLAIN MUSCLE.

V. THE INFLUENCE OF CAFFEINE ON LACTIC ACID FORMATION.

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(*Received May 27th, 1926.*)

The production of rigor in skeletal muscle by the action of caffeine, discovered by Cogswell [1852], has formed the subject of several investigations. Ransom [1911] showed that the rigor is accompanied by lactic acid production, and this was confirmed and the conditions of lactic acid formation further studied by Meyerhof [1921] and by Matsuoka [1924]. The alterations in the lactacidogen content of the muscle submitted to the action of caffeine was studied by Riesser and Neuschloss [1922], whilst the effects on heat production were studied by Hartree and Hill [1923]. From these researches it would seem that the effect of caffeine is greatly to accelerate the breakdown of glycogen and of lactacidogen into lactic acid, by unloosing a chain of processes similar to those which ensue upon stimulation of the muscle; in presence of oxygen, the oxygen usage of the muscle is greatly increased though there does not appear to be any restitution of lactacidogen.

The rigor which is produced in striated muscle by high concentrations of caffeine is irreversible, and is accompanied by destruction of the structure of the muscle fibres, which has been studied by Secher [1914].

In a recent publication [Evans, 1925], I hinted that caffeine did not appear to cause acceleration of lactic acid production in plain muscle, and it is the further investigation of this point which forms the subject of the present communication.

Methods.

Plain muscle from the tortoise stomach was used; the stomachs of several animals were used for each experiment and the sampling and estimations were carried out as described in the former paper [Evans, 1925], except that the tissue was not frozen but was killed by ice-cold 4 % HCl saturated with NaCl.

The estimation of lactic acid by the modified Furth and Charnass method is not interfered with by the presence of caffeine, as the following experiments show.

Exp. 1.

Caffeine taken (mg.)	Lactic acid taken (mg.)	Lactic acid found (mg.)	Recovery %
15	2·45	2·40	98
15	2·45	2·35	96
30	2·45	2·18	89
0	2·45	2·2	90
0	2·45	2·25	92
0	2·45	2·2	90
0	2·70	2·60	96
0	2·70	2·62	97
0	2·70	2·39	89
100	0·0	0·0	—

In order to make quite certain that no effect of any kind was introduced by the presence of caffeine, in some of the experiments caffeine was added in equal amounts to all the samples, either before the experiment began, or after the tissue had been killed by acid. The same results were obtained when this was done as when caffeine was not added to the control samples.

Retardation of lactic acid formation by caffeine.

When portions of plain muscle were suspended in (frog) Ringer's solution containing caffeine, and kept anaerobically (by $N/500$ NaCN or nitrogen), there was always a smaller production of lactic acid in a given time than in control samples in Ringer without caffeine under the same conditions. The inhibition of lactic acid formation varies in proportion to the caffeine concentration. In the following experiment, where there were very small amounts of caffeine, the reduction was not much beyond the amount of the experimental error; but it is important to know that even with small concentrations of caffeine there is never any acceleration of lactic acid production, since some of Meyerhof's figures suggest that, even with striated muscle, the accelerated acid formation at lower concentrations may give place to a retarded rate when the concentration of caffeine is above a certain amount. This would probably be due to the fibres being killed quickly.

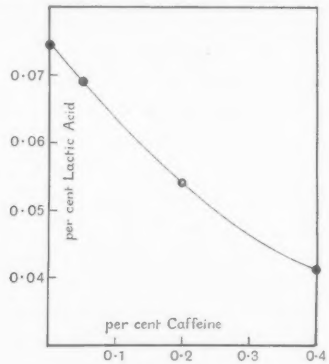


Fig. 1.

Exp. 2. Tissue samples about 3½ g. in 5 cc. of solution at 13°.
Control tissue in Ringer + $N/500$ NaCN for 2 hours gave 0·057 % lactic acid (mean of two estimations).
Tissue in Ringer + 0·002 % caffeine + $N/500$ NaCN for 2 hours gave 0·052 % lactic acid.
Tissue in Ringer + 0·01 % caffeine + $N/500$ NaCN for 2 hours gave 0·054 % lactic acid.

When the amount of caffeine is greater, the inhibition is more pronounced and, as shown by Fig. 1, is roughly in linear relationship to the concentration

of caffeine present, which there varied from 0·05 to 0·4 %. In this experiment the samples of tissue (about 3 g.) were suspended in 5 cc. of the Ringer-cyanide solution for 2½ hours at 16°.

After more prolonged action the inhibition is often still more pronounced as the next experiment shows (see also Exp. 6).

Exp. 3. Samples about 3·5 g. in 10 cc. of Ringer's solution at 12° in atmosphere of nitrogen. Fresh "resting" control, 0·019 % lactic acid.

Period in solution	Lactic acid present in solution: With no caffeine	With 0·3 % caffeine	Inhibition
2·5 hours	0·036 %	0·029 %	20 %
5·0 „	0·058	0·039	33

It is evident, therefore, that the tissue is not killed at an early stage by the caffeine, thus causing the lactic acid production to stop short, and further proof of this will be seen later.

The retardation is seen equally well when the tissue samples are placed in phosphate solutions instead of in Ringer's solution, as was done in Exps. 4, 5 and 6.

Exp. 4. Samples suspended in $M/15$ Na_2HPO_4 solution at 15°, in air, without cyanide. Fresh control, 0·008 % lactic acid.

Duration	Lactic acid in: Phosphate alone	Phosphate + 0·4 % caffeine	Retardation
1 hour 25 minutes	0·047 %	0·034 %	27 %
4 hours	0·064	0·056	12
Exp. 5. Fresh control, 0·026 %.			
30 minutes	0·059	0·043	27
Exp. 6. (In nitrogen) fresh control, 0·027 %.			
1 hour	0·066	0·047	29
2 hours 20 minutes	0·100	0·067	33
4 hours	0·126	0·074	41

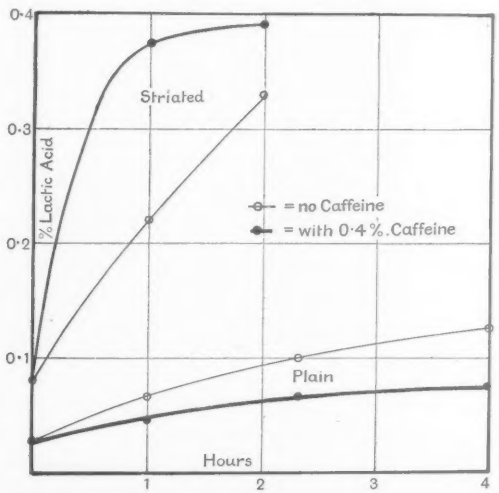


Fig. 2.

In order to exclude the possibility that the muscle of the tortoise is in any way peculiar in its behaviour towards caffeine, an experiment was carried out with plain and striated muscle of this animal with and without caffeine. Samples of about 5 g. of plain, and of about 2 g. of striated muscle were suspended in 10 cc. of $M/15$ Na_2HPO_4 solution, in air, at 17·5°, and the rates of lactic acid formation compared. The results are given in Fig. 2, and show that the striated muscle behaves just like that of the frog and higher animals, in exhibiting acceleration of lactic acid formation in presence of caffeine, whilst the plain muscle shows the reverse.

When placed in phosphate solutions of lower hydrogen ion concentrations, the retardation is still seen and to about the same extent, though in any case the rate of lactic acid formation is definitely greater in the more alkaline than in the more acid solutions, as I have shown previously [Evans, 1925].

Exp. 7. Samples in 5 cc. solution at 15° for 3 hours.
Resting control, 0·023 % lactic acid.

	Lactic acid after 3 hours in	
Phosphate solution	Solution without caffeine	Solution with 0·3 % caffeine
$M/15$ Na_2HPO_4 (p_H 9·0)	0·066 %	0·046 %
$M/15$ NaH_2PO_4 (p_H 4·5)	0·053	0·036

Retardation by caffeine at p_H 9·0 = 30 %.
Retardation by caffeine at p_H 4·5 = 32 %.

It is important to note that addition of caffeine to the solution in which the muscle is kept merely *retards* the formation of lactic acid and does not produce the effect by causing early death of the muscle before it has had time to generate the acid. This is further illustrated by the next experiment, which shows that if sufficient time is allowed to give a lactic acid maximum, then this is the same whether caffeine is present or not.

Exp. 8. Tissue kept in $M/15$ $Na_2HPO_4 + N/500$ NaCN.

	% lactic acid present in tissue kept in		
Time	Solution without caffeine	Solution with 0·3 % caffeine	Lactic acid % difference
0 (control)	0·023	—	—
3 hours	0·066	0·046	0·02
23 hours	0·122	0·119	0·003

Taken together, the experiments so far described indicate quite clearly that in presence of caffeine, the formation of lactic acid in plain muscle under anaerobic, or approximately anaerobic, conditions, is definitely slowed. What now will be the effect of caffeine on the oxidative recovery process in this tissue?

Relation of caffeine to the recovery process.

This question was attacked in the first instance by subjecting portions of tissue to nitrogen for some hours, with or without caffeine, and then studying the effect of removal to oxygen for longer periods, lactic acid estimations being made on samples at each stage. Some results are given in Exps. 9 and 10.

Exp. 9. Fresh control, 0·019 % lactic acid. Samples of the tissue suspended in Ringer's solutions, without and with 0·3 % of caffeine, were exposed to nitrogen at 12°. The tissue without caffeine, after 5 hours in N, contained 0·058 % lactic acid, whilst the sample with 0·3 % caffeine contained 0·039 % lactic acid. The tissues were then transferred to oxygen, with shaking (not very efficient) for 18 hours, after which the sample with no caffeine contained 0·052 %, and that with caffeine 0·045 % lactic acid.

There was thus a slight actual reduction of lactic acid in absence of caffeine, whilst in presence of caffeine there was a slight increase. One must remember, however, that this result might be compatible with the caffeine leaving the recovery process fundamentally unaffected, the slight difference being due to the greater velocity of the recovery process in absence of caffeine because of the higher initial concentration of lactic acid in that instance. If, therefore, the recovery processes were normal the difference in the final figures would be due to the natural acceleration of the process in the first instance.

In the next experiment an attempt was made to test this hypothesis.

Exp. 10. Samples of fresh tissue were exposed in the moist state to moist nitrogen, without addition of caffeine, for 3 hours at 15°. The initial content of lactic acid was 0·023 %, and that after 3 hours' exposure to nitrogen was 0·063 %. One sample was now transferred to 5 cc. of oxygenated Ringer, and kept in oxygen with occasional agitation for 20 hours. Another sample was similarly treated with oxygenated Ringer containing 0·3 % of caffeine, and exposed to oxygen also for 20 hours. The lactic acid contents were then found to be:

(*a*) Without caffeine = 0·082 %.
(*b*) With caffeine = 0·087 %.

In both cases there was therefore an increase in the lactic acid content, though not to such a high level as if oxygen had been withheld. The results of the experiment appear somewhat doubtful, but again suggest that the recovery process has not been greatly affected by the presence of caffeine.

The next experiment was made by comparing the lactic acid formation in presence of caffeine under anaerobic conditions with that under reasonably good conditions for oxygen access.

Exp. 11. Each sample (of about 4·5 g.) of tissue was suspended in 5 cc. Ringer's or phosphate solution containing 0·3 % of caffeine. One series was placed in bottles filled with oxygen, and was shaken briskly for 2½ hours at 14·5°. To the other series *N*/500 NaCN was added, and the sample left at rest also for 2½ hours at 14·5°. The results follow:

	Lactic acid present in tissue treated with	
Tissue in	Oxygen and caffeine	Cyanide and caffeine
Ringer (fresh control)	(0·024) %	(0·024) %
Ringer 2·5 hours	0·025	0·029
NaH_2PO_4 2·5 hours	0·025	0·041
Na_2HPO_4 2·5 hours	0·024	0·039

The experiment shows that when oxygenation is efficient, the lactic acid can be kept down to its initial value when caffeine is present, though in absence of oxygen respiration, there is, in spite of the presence of the caffeine, a definite increase. This experiment therefore also suggests that the recovery process is either accelerated slightly, which would not accord with the two previous experiments, or is unaltered by caffeine.

The clearest indication that the processes of oxidative recovery are unaltered by caffeine is obtained by observing the oxygen usages of the tissue

with and without caffeine, and comparing these with what happens with the tortoise striated muscle. The results of such an experiment are given in Fig. 3.

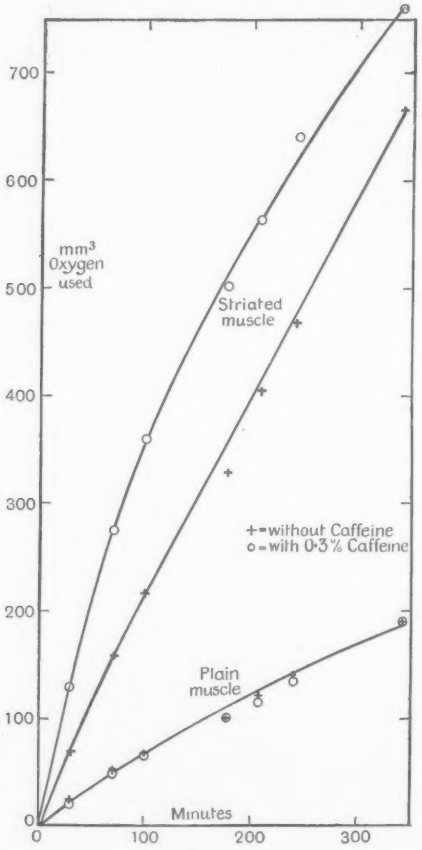


Fig. 3.

This figure shows the results of a microrespirometer experiment, the total oxygen usages being plotted against the time. The effect of caffeine on the tortoise striated muscle is precisely similar to that found on the frog's striated muscle by Meyerhof, namely, a large increase in the oxygen utilisation, particularly during the earlier periods, as the slope of the curve shows. On plain muscle, there appears to be no effect at all; at the end of 342 minutes the total oxygen used per g. at 15° was, in presence of caffeine 189 mm.^{3}, and without caffeine 190·5 mm.^{3}; at all intermediate times also, the amounts were as nearly identical as could be expected.

Action of arsenate and caffeine.

I have shown in the previous publication that arsenates accelerate lactic acid formation in plain muscle, just as Meyerhof found them to do in striated muscle.

It was therefore of interest to know how caffeine and arsenate in conjunction would act on plain muscle.

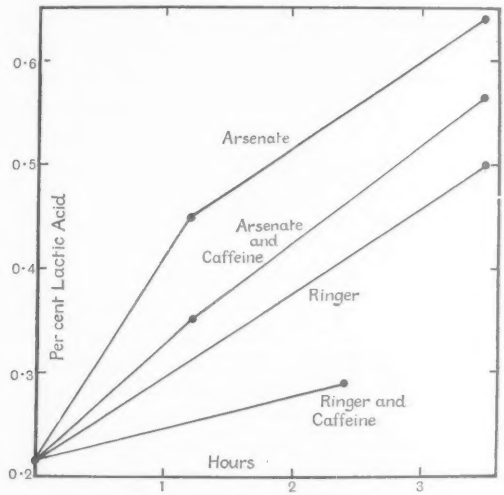


Fig. 4.

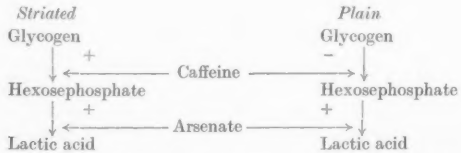
Fig. 4 gives the result of an experiment in which portions of plain muscle were treated with Ringer's solution containing (*a*) 0·3 % sodium arsenate, (*b*) 0·3 % caffeine, (*c*) 0·3 % arsenate plus 0·3 % caffeine, (*d*) neither addition. The results shown in the figure prove that caffeine can exert a decidedly inhibitory effect on the arsenate.

Discussion.

The difference in the action of caffeine on plain and on striated muscle is of some theoretical interest, particularly in view of the identical action of arsenates on the two tissues. If we assume that arsenate plays some rôle in accelerating the breakdown of hexosephosphate, as it does in fermentation and apparently in striated muscle, then it seems reasonable to suppose that it may play a similar part in plain muscle. But since caffeine has an opposite effect on the two varieties of muscle, it would appear that it does not act on the same link in the chain of reactions as arsenate does, but that the action is of a physico-chemical nature in the first instance and, in striated muscle, depends on its causing structural damage to the tissue and so starting the breakdown process further back than arsenate does. The investigations of Hoet and Marks [1926] have recently given grounds for the belief that the

onset of rigor (and perhaps of contraction too) in striated muscle depends upon the depletion of glycogen, and it would not be going far to postulate, as the underlying cause of contraction, the disturbance, in a reversible manner, of a state of physico-chemical equilibrium in which glycogen plays a part, the formation of lactic acid being a collateral and perhaps a synergic phenomenon. I have shown [Evans, 1926] that glycogen is the lactic acid precursor in plain as it is in striated muscle.

Secher found, in investigating the action of caffeine on the histological structure of muscle, that it had no effect on the unstriated muscles of the invertebrates. The destructive action therefore is absent. Yet caffeine *delays* lactic acid production, even that which is occasioned by the presence of arsenates. This suggests that the physical or chemical conditions of equilibrium in which glycogen is held may be different in the two forms of muscle, and in support of this view is the fact that even after depletion of its glycogen, plain muscle does not pass into rigor mortis. Disturbance of an equilibrium in which glycogen is concerned would thus perhaps appear to be less probable as an explanation of contraction in plain than in striated muscle, the evidence of the present paper so far being rather in favour of the lactic acid hypothesis in its direct form. Caffeine retards the spontaneous breakdown or liberation of the lactic acid precursor in plain muscle, and thus forestalls the possibility of formation of lactic acid, even when arsenate is there to accelerate reactions further on in the chain.



Finally, a few experiments which I have carried out show that caffeine causes relaxation of the tonus and cessation of movements of plain muscle, and also abolishes the contraction produced by such drugs as histamine, whilst arsenates cause contraction. It might be expected that other drugs which cause powerful contraction of plain muscle would also accelerate the lactic acid production, but up to the present I have not found this to be so with histamine or acetylcholine.

Summary.

1. In presence of caffeine, in concentrations from 0·002 to 0·4 %, the rate of lactic acid formation in plain muscle under anaerobic conditions is diminished.

2. The retardation of lactic acid formation is present in phosphate solutions of p_H 4·5 and p_H 9·0. The effect is merely a delay, since ultimately the same lactic acid maximum is reached whether caffeine be present or not.

3. The oxidative recovery process is not affected by caffeine; nor is the oxygen usage of the fresh tissue altered.

4. In presence of caffeine, the effect of arsenates in accelerating lactic acid formation is reduced. The effect of caffeine is probably physical or physico-chemical.

The cost of the experiments was, in part, defrayed out of a grant from the Government Grants Committee of the Royal Society.

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CXIII. A PRELIMINARY STUDY OF FACTORS INFLUENCING CALCIFICATION PROCESSES IN THE RABBIT.

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(*Received July 26th, 1926.*)

Early in 1918 E. Mellanby published a preliminary report of his experimental work on rickets in puppies, emphasising the importance of a vitamin similar to vitamin A in the development of the disease. This calcifying vitamin has recently been called vitamin D. Since 1920 much work has appeared on the subject, the majority of the additional workers using rats as their experimental animals. Arising out of E. Mellanby's work one of us (M. M.) has been for the past eight years investigating the factors influencing the development of teeth and jaws in puppies and other animals with the object of attempting to discover the main causes of the bad teeth of civilised nations. It was shown that foods contain two factors potently affecting the formation of the teeth and their reaction to harmful stimuli. One of these is the calcifying vitamin and the other, acting in the opposite way, some factor present in cereals [M. Mellanby, 1918, 1920, 1923, 2].

This work has also been extended in two ways to children.

(1) A large number of deciduous teeth of children have been examined and it was found

(*a*) that they were badly calcified, to the extent of over 80 %, not 3 % as usually stated [M. Mellanby, 1923, 1];

(*b*) that there is a direct relationship between structure and caries, in general the worse the structure the more the caries [1923, 1];

(*c*) that, when teeth are well calcified and yet carious, their resistance after eruption must have been poor as evidenced by the badly formed or deficient secondary dentine; and, *vice versa*, when teeth are badly formed and yet non-carious, the secondary dentine is abundant and well calcified, indicating good resistance. Inferring from the results of the experimental production of secondary dentine in puppies, it seemed probable that, at the time when the noxious influences affected the children's teeth, bad or good secondary dentine, respectively, was formed owing to the diet at the time being on the one hand defective in calcifying powers and on the other potently calcifying [1923, 3].

(2) On the basis of the above results an investigation was undertaken by M. Mellanby, Pattison and Proud [1924] to test the effect of diet on caries in

erupted teeth of children. The children were divided into three groups, the diets of which varied in their calcifying powers as estimated by the animal experiments.

Group A had a potently calcifying diet,

,, B had a much less potently calcifying diet,

,, C had an intermediate diet as regards calcifying properties.

It was found that the spread of caries was nearly three times greater in group B than in group A, and intermediate in group C. From all these facts it may be inferred

(*a*) that well and badly calcified teeth can be produced at will by altering in the diet the relative amounts of calcifying vitamin, found in milk, egg-yolk, cod-liver oil, etc., and anti-calcifying substances found chiefly in cereals,

(*b*) that the better calcified the teeth the less liable are they to be attacked by caries, and

(*c*) that after eruption a calcifying diet tends to increase the resistance of the teeth, whatever their structure, to the onset and spread of caries.

To put all these inferences to a final test it is essential that experimental caries should be capable of production at will in animals, for it is difficult by any other means than the animal experimental method to control all the factors except the one to be tested.

For a long time experiments have been carried out by one of us (M. M.) principally on dogs with little or no success as far as experimental caries *in vivo* is concerned. McCollum, Simmonds, Kinrey and Grieves [1922] in America claim to have produced "caries-like" lesions in the teeth of rats, but as these animals are very small, and as so far we have not succeeded in reproducing the results obtained by the American workers, we sought for another experimental animal. We had heard privately from Sir Frank Colyer that rabbits sometimes suffer from caries under domestication and we therefore determined to make use of these animals in spite of the fact that their molars are of the persistently growing type.

Up to the present time we have not seen caries in any of the experimental animals, but we have now obtained diets which will allow the animals to live for periods up to one year and yet which might be expected on the basis of the work above mentioned to lead ultimately to caries, for the teeth and bones of animals fed on these diets are poorly calcified. A drawback to the rabbit, which applies also to the rat, is that it is impossible to examine the molars during life so that the condition of these teeth can only be determined *post mortem*. In the meantime it was thought worth while to publish the results up to date as they may prove useful to other investigators working on the calcification processes in animals and especially on the subject of rickets and the anti-rachitic effect of foods and ultra-violet radiations.

Using the puppy experiments and the investigations on children as a basis, the first object was to find a diet deficient in the calcifying vitamin and yet so closely resembling the usual diet of these animals when under domestication

that they would eat it readily. A mixture consisting of four parts of oats to one part of bran, plus 1·5 % $CaCO_3$ and 6 cc. decitrated orange or lemon juice, proved to be a suitable basal diet; animals on this diet usually grow fairly well and develop rickets (Plate III, 1 B) and badly calcified teeth (Plate V, 3 B). Papers have recently appeared by Goldblatt and Moritz [1925], who have produced rickets in rabbits by feeding them on diets built up on the lines found to give rickets in rats; the diets used by these workers, however, were somewhat artificial and the duration of the experiments short. The feeding methods described below appear to exclude these two drawbacks, for the foods are more natural to the animals and consequently they live for periods up to at least one year. Other workers [Nelson and Lamb, 1920] have shown that rabbits fed on a diet deficient in fat-soluble vitamin A, for periods of 60 days or more, develop xerophthalmia. Some degree of xerophthalmia has been noticed in only four cases on the above-mentioned basal diet. Probably this slight incidence may be explained by the fact that our diets were not composed of purified foodstuffs and therefore even the basal diet was not free from fat-soluble vitamin A. Nelson and Lamb do not state whether they observed any symptoms of rickets in their animals. A summary of the results now to be described was given before the Physiological Society on Feb. 20th, 1926 [M. Mellanby and Killick, 1926].

Experimental Methods.

Litters of rabbits were always used in comparative experiments. The special diet was usually started when the animals were about eight weeks old and weighed from 350–650 g., according to the breed. Each experimental animal was kept in a separate cage in a moderately well lighted room. The cages were of galvanised iron wire, so that they could easily be sterilised. The animals got very little exercise, except in two experiments in which the effect of exercise was tested, as the cages were small. The room temperature, particularly for young rabbits, was found to be of some importance, the most suitable appearing to be about 55° F. When the temperature fell much below 50° F., the young animals frequently developed diarrhoea and died. As an additional safeguard against cold, the litters were kept in wooden hutches until they were required for experiment, and, in the early days of the experimental period, they were put together at night. Daily notes were made of any special features seen. The animals were weighed twice a week; radiographs of each experimental series were taken when the first clinical signs of rickets or scurvy appeared in any member of the series, and in some cases, at intervals throughout the experiment.

Diagnosis of the state of bones and teeth was made by:

(1) clinical signs during life;
(2) radiographs of left foreleg during life and of jaws at death;
(3) *post mortem* findings;

(4) histological structure (decalcified and undecalcified sections of rib, radius, ulna and teeth);

(5) estimation of Ca in the femur shaft.

In this publication the majority of illustrations and other data given are those of the bones because differences are more easily seen. The changes in teeth and jaws (Plate V, 3 and 4) run parallel.

Feeding. In the earlier experiments the animals were allowed to eat as much dry oats and bran as they would, and only the variable in the diet was regulated in amount. The results obtained in this way were of course not strictly comparable, as the degree of rickets in animals fed on a vitamin-deficient diet depends partly on the rate of growth [E. Mellanby, 1920]. When, therefore, a suitable basal diet for producing badly calcified teeth and bones had been found, all the food given was, as far as possible, either weighed or measured for each animal. Special ingredients of the diet, such as lemon juice, any oils or fats, yeasts, etc., were measured and mixed with the oats and bran. The usual custom in the later experiments was to give water, equal in weight to the dry part of the diet. The water balance was found to be of importance to the health of the animals.

All the animals of one series were given the same quantity of food and water; any leavings were recorded, so that the amount could be regulated, if so desired, by the animal of the litter eating the least food. In most cases, any vegetable to be eaten was given as an extra in addition to the amount of other food. In several cases, however, those animals which had vegetable *ad lib.* were given a smaller amount of oats and bran than the others, the amount being regulated so that the rate of growth in all members of the series was as nearly the same as possible.

The experiments were complicated at first by several cases of scurvy, three animals showing definite and extensive haemorrhages at the *post mortem* examination when fed on oats, bran and a small quantity of grass. Two of them had winter grass, the third had heated and oxygenated grass. A fourth, fed on oats, bran and 20 g. cabbage, showed definite haemorrhages at *post mortem*. Symptoms of scurvy were sometimes noted clinically and also by radiographs taken during life; the diagnosis was also confirmed histologically after death. Other animals fed on an intentionally scorbutic diet showed varying results. Two developed definite scurvy; the majority showed some indications of the disease, while a few developed no such symptoms.

Rabbits appear to be on the border-line between those animals, such as guinea-pigs, which develop scurvy readily when fed on a diet deficient in antiscorbutic vitamin, and those much less sensitive to a lack of it, such as the rat. In order, therefore, to study rickets and allied teeth conditions, uncomplicated by possible scorbutic changes, some antiscorbutic substance had to be included in the basal diet. White turnip proved to be very successful as an antiscorbutic, improving the appetite and general health of the rabbits, and showing a complete lack of calcifying power, but, owing to the difficulty

in obtaining a continuous supply of this vegetable, it was thought more suitable to use lemon juice, decitrated by Zilva's method, as a standard.

The mixture of four parts oats to one part bran with 6 cc. of decitrated lemon juice was first adopted as the basal diet. Rabbits fed on this diet did not eat much after the first few weeks. They soon ceased to grow, then lost weight and died. The period of life varied from $2\frac{1}{2}$ to 11 weeks. All the animals showed some signs of rickets, although in the majority of cases it was very slight (see Plate III, 2 A), the actual degree varying with the rate of growth and the length of time the animal was on the diet.

With the object of improving the diet with respect to the Ca : P ratio, 1·5 % $CaCO_3$ was added to the oats and bran mixture. The Ca : P ratio, calculated from figures given by Sherman [1924], was thus altered from 0·08 : 0·55 to 0·68 : 0·55, a ratio very much closer to the optimal for good calcification as given by McCollum for rats. The effects of this addition were very striking; the animals ate better, grew better and lived much longer, but developed badly calcified bones and teeth. Plate III, 2, shows the radiographs of the forelegs of two rabbits of the same litter, *A* and *B*, one without (*A*) and one with $CaCO_3$ (*B*). From the weight curves (Fig. 1) and radiographs (Plate III, 2) it will be seen that the animal receiving $CaCO_3$ ate more and grew better and had worse rickets than the one without this salt.

Table I. *The effect of adding $CaCO_3$.*

Age at beginning of experiment: 9 weeks.
Weight at beginning of experiment: 430–500 g.
Time of year at beginning of experiment: December.
General diet: Rolled oats 4, Bran 1, Lemon juice. } *ad lib.*

No. of exp.	Variable factor	Time on diet weeks	Gain in weight g.	Calcium % wet weight	Calcium Amount g.	X-ray	Histology
65	No $CaCO_3$	$6\frac{1}{2}$	130	11·4	0·097	Slight R.	Slight R.
67	No $CaCO_3$	11	200	9·6	0·101	,,	,,
68	1·5 % $CaCO_3$	17	420	13·1	0·130	Bad R.	Rickets
69	1·5 % $CaCO_3$	$11\frac{1}{2}$	360	10·9	0·150	,,	,,
70	1·5 % $CaCO_3$	17	450	13·24	0·117	,,	Bad R.

It will be seen from Table I that, although the bones of the two animals with no $CaCO_3$ showed signs of only slight rickets when examined macroscopically or microscopically, yet their low Ca content indicated that the defect in calcification was there, but was not manifest because of the failure of the animals to grow well. This suggested that with a deficiency of calcifying vitamin and excess of oats, the degree of rickets which developed depended mainly upon the rate of growth of the animal, and that the effect of the added $CaCO_3$ in causing bad rickets was largely a growth effect [E. Mellanby, 1918, 1919].

To test this hypothesis, two animals were fed on oats, bran, 1·5 % $CaCO_3$ and lemon juice. Rabbit 168 was given as much of the mixture as it could

eat—$2x$ g. daily, while rabbit 167 was given only half this quantity, x g. There was naturally a great difference in the rates of growth of these animals (Fig. 2). The weight curves during the first 9 weeks of the experiment will be seen to be very similar to those of the animals with and without $CaCO_3$ (Fig. 1). The rabbit eating $2x$ g. of the mixture and growing the more rapidly developed bad rickets, whilst the one having x g. showed only very slight rickets.

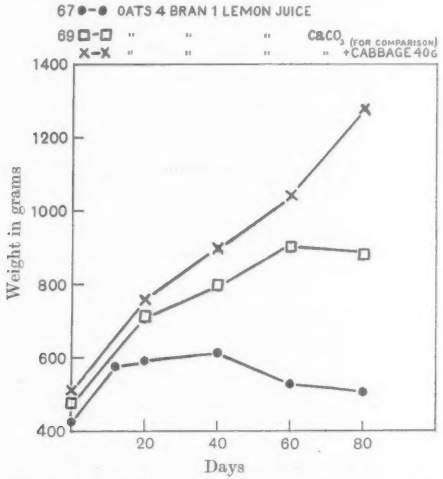

Fig. 1. Weight curves showing the improvement in growth resulting from adding $CaCO_3$ to the basal diet and the further improvement on adding cabbage. All these animals developed rickets.

Table II. *The effect of doubling the amount of basal diet.*

Age at beginning of experiment: 8½ weeks.
Weight at beginning of experiment: 580–650 g.
Time of year at beginning of experiment: July.
General diet: Rolled oats 4, Bran 1, Lemon juice. } 1·5 % $CaCO_3$.

No. of exp.	Variable factor g.	Time on diet weeks	Gain in weight	Calcium % wet weight	Calcium Amount g.	X-ray	Histology
167	x	15	—	14·2	0·141	Slight R., healing at death	Nearly normal
168	$2x$	15		14·4	0·159	Bad R.	Rickets

These results emphasise the importance of controlling the quantity of food actually eaten by each of the animals of a given series, and of keeping their rates of growth as nearly the same as possible.

Out of a total of 22 animals fed on the basal diet now used (oats 4, bran 1, plus 1·5 % $CaCO_3$ and lemon juice) 19 had definite rickets; two developed osteoporosis; one, however, had apparently normal bones and teeth, making

it the one unexplained exception to the general result. Thus it appears that if a healthy, normally growing young rabbit is given this basal diet it eats moderately well and develops rickets within 7 weeks, and sometimes as early as 10 days according to its rate of growth and probably also to its antenatal history.

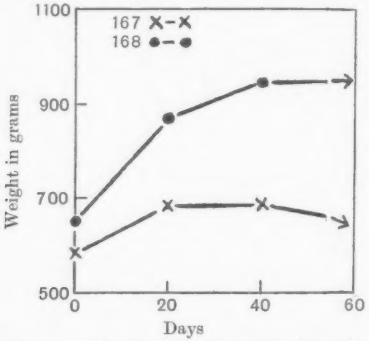

Fig. 2. Weight curves showing the effect of doubling the basal diet. The rickets developed by *A*, 168 (2*x* g. diet) was much greater than that developed by *B*, 167 (*x* g. diet).

The results with regard to bone changes were fairly constant, and it has also been found that animals on this rickets-producing basal diet develop poorly calcified alveolar bone and badly formed dentine containing many interglobular spaces (photomicrograph and radiograph, Plate V, 3 B and 4 B).

THE CALCIUM AND PHOSPHORUS CONTENT OF THE RICKETS-PRODUCING DIET.

Whereas it is true that the normal calcification of bones and teeth can only be produced when the diet contains a minimum of calcium and phosphorus, it is equally certain that these necessary minima depend upon other factors, the more important of which are:

(1) the amount of calcifying vitamin eaten,

(2) the rate of growth,

(3) the amount of exposure of the animal or certain food constituents to ultra-violet radiations,

(4) in some (*e.g.* dog) and possibly all animals the amount of some anti-calcifying substance in cereals.

It will be seen later that the calcifying vitamin prevents rickets and allows the production of normally developed teeth and jaws in rabbits as in puppies; and it may be said with some certainty that, if there is abundance of anti-rachitic vitamin in the food, the question of calcium-phosphorus ratio and balance is negligible in questions of the aetiology of rickets and defective tooth calcification, and that even the absolute amounts of calcium and phosphorus in the diet, so far as ordinary foods are concerned, are of but small importance. The calcium and phosphorus retaining powers possessed by the calcifying vitamin of cod-liver oil are so great as to make it almost impossible

to produce abnormality of calcification of bones and teeth by ordinary foods in the presence of this oil. The question we have to deal with now, however, is the importance of these elements when there is a deficiency of calcifying vitamin in the food eaten.

The effect of the rate of growth on calcification has been seen above to be one of great importance. If x g. of a certain diet produce a small amount of rickets, $2x$ g. of the same food, containing twice the calcium and phosphorus intake, make the rachitic condition worse when all other factors are equal. It is very probable that the increase in rickets is due to the greater rate of growth. Therefore, even if there were no factors influencing the development of rickets other than growth, it is obviously impossible to assign great importance to the calcium-phosphorus ratio of the diet in the aetiology of this disease, for this ratio is the same whether $2x$ or x g. are eaten.

So much importance has, however, been attached to the questions of the calcium-phosphorus intake in relation to rickets, especially by American investigators, that this aspect of the problem deserves further consideration in the light of the experimental work on rabbits. It is undoubted that, when the diet is deficient in calcifying vitamin, the amount of calcium and phosphorus ingested bears some relation to the ultimate calcification of bones and teeth. The improvement in bone and teeth formation resulting from the addition of calcium carbonate to a diet deficient in calcifying vitamin, and especially when butter formed a constituent of the mixture eaten, has been described [E. Mellanby, 1921]. Again, Elliott, Crichton and Orr [1922] found that rickets in pigs could be prevented by adding a salt mixture containing calcium phosphate, calcium carbonate, magnesium sulphate, potassium carbonate, ferric chloride, sodium chloride and sodium hydroxide to a diet of oatmeal, rice and skimmed milk.

The following table, slightly modified from that of Goldblatt [1924] represents the calcium and phosphorus contents and ratios in some of the standard diets used by the respective workers for producing defective bone formation in rats and that described above for rabbits:

Table III.

	Diet	Animal	Effect on animal	Calcium %	Phosphorus %	Ca : P ratio
Sherman and Pappenheimer [1921]	84	Rat	Rickets	0·553	0·087	1 : 0·15
McCollum, Simmonds, Shipley and Park [1922]	3143	,,	,,	1·231	0·301	1 : 0·25
McCollum, Simmonds, Becker and Shipley [1922]	3407	,,	,,	1·202	0·217	1 : 0·18
Korenchevsky [1921]	−A −P	,,	Osteoporosis	0·256	0·22	1 : 0·86
Goldblatt [1924]	−A −P +$CaCO_3$	,,	Rickets	1·08	0·22	1 : 0·20
Chick and Roscoe [1926]	F.R.	,,	Osteoporosis	0·27	0·37	1 : 1·37
M. Mellanby and Killick [1926]	—	Rabbit	Rickets	0·67	0·550	1 : 0·81

The optimum amounts of calcium and phosphorus for normal bone formation in rats has been described by McCollum as 0·75 % and 0·59 % respectively,

or a ratio of 1 : 0·67. One of the main features, therefore, about the diets mentioned in Table III and used in the rat-feeding experiments for producing rickets would seem to be the low phosphorus intake and especially the small amount of phosphorus as compared with the calcium. A ratio nearer that given as optimal for calcification in rats is seen in Korenchevsky's – A – P diet [1921]. This diet does not regularly produce definite rachitic changes but results in osteoporosis. Again, the Chick and Roscoe diet [1926] has a Ca-P ratio of 1 : 1·37 and produces osteoporosis and not rickets. Goldblatt [1924] suggested that this lack of regularity in rickets production was due to the ratio between the calcium and phosphorus intake, for, when calcium carbonate was added to the – A – P diet of Korenchevsky so as to change the Ca : P ratio from 1 : 0·86 to 1 : 0·20, rickets was readily produced. At first sight it might appear that the 1·5 % $CaCO_3$ in the standard diet for producing poor calcification in rabbits is comparable to the addition of $CaCO_3$ by Goldblatt to Korenchevsky's diet. This, however, does not appear to be the case, for the Ca : P ratio in our diet is 1 : 0·81; this resembles the ratio 1 : 0·86 of the original – A – P diet of Korenchevsky which does not regularly produce rickets in rats. In other words, the ratio of calcium to phosphorus which has been found to be effective in producing rickets in rabbits is nearer the optimum said to prevent rickets in rats (*i.e.* 0·75 % Ca, 0·5 % P, ratio 1 : 0·67).

If the Ca : P ratio of the basal rickets-producing diet for rabbits be varied by adding less calcium carbonate than 1·5 %, then the conditions do not appear to be as good for producing growth. When, for instance, 0·75 % $CaCO_3$ is added to the diet, the growth of rabbits is less and the bones, although osteoporotic, are less rachitic. The addition of 3 % $CaCO_3$ instead of 1·5 % also apparently brings about a slower rate of growth, but nevertheless the bony changes are usually now of a severe rachitic nature.

The point might be raised that, although the optimum Ca : P ratio for producing rickets in rabbits under these conditions is such as tends to prevent the disease in rats, yet the absolute amount of these elements eaten may be low. This, however, does not appear to be the case, for the percentages of Ca and P in the diets which are well eaten and allow good growth in the rabbits are 0·67 % and 0·55 % respectively. These figures are high compared with the figures 0·256 % and 0·22 % of Korenchevsky's – A – P diet, and are roughly comparable with those said by McCollum to be required for normal calcification in rats.

If 1·55 % $Ca_3(PO_4)_2$ be added to the oats and bran mixture instead of 1·5 % $CaCO_3$ the calcium content of the diet remains constant, but the additional phosphorus changes the Ca : P figures from 0·67 : 0·550 or 1 : 0·81 to 0·67 : 0·86, or 1 : 1·28. In this case the animal grows as well as when the diet contains $CaCO_3$, but the calcification appears much improved (Table IV).

Acid-base ratio of the ash.

The importance of the acid-base ratio of the ash of the diet in the production of rickets has been considered by various workers, including McCollum, Simmonds, Shipley and Park [1922], Zucker, Johnson and Barnett [1923] and E. Mellanby [1925], but no general agreement on this subject has been obtained. Some experiments were therefore made on rabbits to test the acid-base hypothesis by substituting sodium carbonate and sodium phosphate respectively for the calcium carbonate. Under the experimental conditions, however, only poor growth resulted, and, whether sodium carbonate or sodium phosphate (Na_2HPO_4) was added, an intense and thick band of calcification was produced at the growing epiphyseal ends of the bones (see Plate III, 3; Exp. 340, Table IV). The condition is apparently the opposite to that of rickets, calcification being more powerful than growth. In some ways this condition simulates the changes seen in achondroplasia.

Further work on the Ca : P ratio and other mineral constituents and their relation to calcification is necessary before any conclusions can be reached. At present, however, it can be said that the optimum Ca : P ratio for bad calcification in rabbits is a figure approximating to that which is said to be best for antagonising the development of this disease in rats. It also happens that the growth of young rabbits is good under the experimental conditions when the diet contains the said quantities of calcium and phosphorus and that this is probably a determining factor.

Table IV. *The effect of adding various salts to the diet.*

Age at beginning of experiment: about 7 weeks.
Weight at beginning of experiment: 240–380 g.
Time of year at beginning of experiment: May.
General diet: Crushed oats 4 / Bran 1 } *ad lib.*
6 cc. neutral lemon juice.

No. of exp.	Variable factor	Time on diet up to radiographs weeks	Gain in weight g.	X-rays
337	1·5 % $CaCO_3$ + unsaponifiable substances from 1 cc. cod-liver oil	10	380	Normal
339	5·37 % Na_2HPO_4, $12H_2O$	10	40	Dense band calc.
340	1·6 % Na_2CO_3	10	60	Very dense band calc. (Plate III, 3)
342	1·5 % $CaCO_3$	10	340	Rickets
343	1·55 % $Ca_3(PO_4)_2$	10	340	Very slight rickets

It will be seen from the above table that with Na_2CO_3 (340) and Na_2HPO_4 (339) the animals scarcely ate any food and put on very little weight. In both cases there was a dense band of calcification at the top of the diaphysis (see Plate III, 3). The other three animals put on weight at an equal rate: 342, with $CaCO_3$ developed rickets, 343 with $Ca_3(PO_4)_2$ had only very slight rickets, whilst 337 with $CaCO_3$ and the unsaponifiable substances from 1 cc. of cod-liver oil was normal in every way.

THE CALCIFYING ACTION OF SOME FOODS IN THE CASE OF RABBITS.

1. *Foods rich in calcifying vitamin.*

(*a*) *Cod-liver oil.* Having found a diet which resulted in poor calcification, the next problem was to see what additions to the diet would produce normal calcification of teeth and bones. It proved at first difficult to adapt the basal diet so as to give normal calcification, together with good growth and general health. The common method now used in other animals of adding to the basal diet some fat rich in fat-soluble vitamin is open to objection in the case of rabbits, since these animals do not digest fat easily. When 2 cc. *per diem* of fat is included in their diet their general health is impaired after some weeks. For instance, rabbits given the basal diet plus 2 cc. cod-liver oil *per diem* grew at first at about the same rate as control animals on the basal diet only, but showed no symptoms of rickets (see Plate III, 4). After a period of 7–8 weeks, however, the animals having cod-liver oil suffered a loss of appetite, their weight went down rapidly and, if not killed, they died (Fig. 3). These animals showed no constant lesion of the intestinal tract, although a larger proportion on this diet than on any other were found to have gastric ulcers.

Table V. *The effect of adding cod-liver oil to the basal diet.*

Age at beginning of experiment: 8½ weeks.
Weight at beginning of experiment: 380–480 g.
Time of year at beginning of experiment: May.
General diet: Oats 4, Bran 1 } 1·5 % $CaCO_3$, *ad lib.*

No. of exp.	Variable factor	Time on diet weeks	Gain in weight g.	Calcium in femur shaft: % wet weight	Calcium in femur shaft: Amount g.	X-ray: Bones	X-ray: Teeth	Histology: Bones	Histology: Teeth
127	Turnip, 20 g.	7½	280	12·9	0·091	Bad rickets	Calc. imperfect	Bad rickets	Calc. poor
130	Turnip, 20 g. Cod-liver oil, 2 cc.	7½	315	—	0·161	Normal	Calc. good	Calc. good	Normal
131	Orange juice, 6 cc.	7½	180	12·1	0·093	Rickets	Calc. imperfect	Rickets	Calc. poor
132	Orange juice, 6 cc. Cod-liver oil, 2 cc.	7½	165	13·4	0·116	Normal	Calc. good	Calc. good	Normal

Table VI. *The effect of cod-liver oil with less $CaCO_3$.*

Age at beginning of experiment: 8 weeks.
Weight at beginning of experiment: 600–620 g.
Time of year at beginning of experiment: February.
General diet: Oats 4, Bran 1 } 0·75 % $CaCO_3$, *ad lib.*
6 cc. neutral lemon juice.

No. of exp.	Variable factor	Time on diet weeks	Gain in weight g.	Calcium in femur shaft: % wet weight	Calcium in femur shaft: Amount g.	X-ray	Histology
83	—	13	240	10·7	0·144	Some rickets	Calcification abnormal
84	Cod-liver oil, 2 cc.	8½	270	13·1	0·197	No rickets	Calcification good

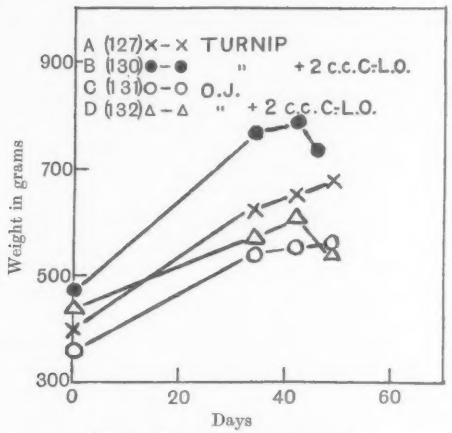

Fig. 3. Weight curves of 4 rabbits.

A received turnip in addition to basal diet (127).
B received turnip + 2 cc. cod-liver oil and basal diet (130).
C received orange juice and basal diet (131).
D received orange juice, cod-liver oil and basal diet (132) (B and D).
Note loss of weight in both animals receiving cod-liver oil after 6 weeks.

The method of administering the cod-liver oil did not influence these results, as it was varied in different experiments, as follows: (1) by mouth separately, (2) mixed with a small quantity of oats and bran, (3) mixed with the whole bulk of the food, in each case with the same result. 1 cc. of cod-liver oil *per diem* in general acts in a similar way to 2 cc., but when only 0·5 cc. of the fat is given the animals live longer; the appetite of the animals, however, is not good and after a period of about 2 months they stop growing and finally die at the end of about 4 months. When the unsaponifiable fraction from 1 cc. of cod-liver oil is used as the source of the calcifying vitamin, growth is slightly better and the general health is certainly improved. Four animals on this diet are still alive and going up slowly in weight; one has been on the diet for 6 months. In all cases in which cod-liver oil or the unsaponifiable substance of cod-liver oil has been added to the basal diet the calcification is very good.

When 20 g. or more of green cabbage are added to the basal diet as well as small quantities of cod-liver oil, conditions are much improved; growth, general health and calcification all seem excellent whether 0·5 cc. of cod-liver oil is given as such (Plate IV, 2 D) or whether the unsaponifiable substance from 1 cc. of the oil is given (Plate IV, 2 C). Animals have been kept alive and are still alive on these diets showing constant increase in weight for over 6 months (Fig. 4) and the calcification of their bones and teeth is very good.

Table VII. *The effect of adding cod-liver oil or its unsaponifiable fraction together with cabbage to the basal diet.*

Age at beginning of experiment: 7½ weeks.
Weight at beginning of experiment: 450–530 g.
Time of year at beginning of experiment: February.
General diet: Crushed oats 4, Bran 1 } 1·5 % $CaCO_3$.
6 cc. neutral lemon juice.

No. of exp.	Variable factor	Time on diet up to radiograph weeks	Gain in weight in 21 weeks g.	X-ray	Notes	
291	Savoy cabbage up to 45 g. Mixture up to 70 g.	21	775	Very bad rickets	Alive after 6 mths.	
292	As 291 plus unsaponifiable substance 1 cc. cod-liver oil	21	1100	Normal	do.	do.
294	As 291 plus 0·5 cc. cod-liver oil	21	1050	Normal	do.	do.

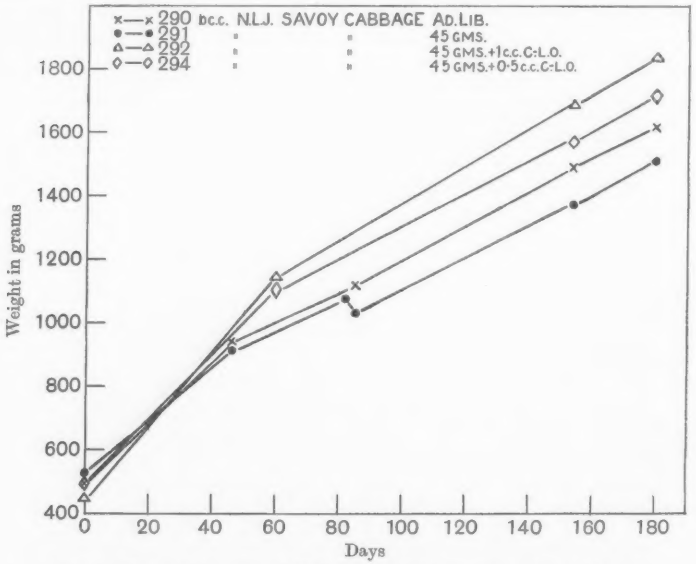

Fig. 4. The weight curves of 290, 291, 292, 294. Radiographs are shown in Plate IV, 2. Note the parallel rate of increase in weight.

(*b*) *Egg-yolk.* The addition of egg-yolk to the basal diet had similar results to those following the addition of cod-liver oil. When one-quarter of an egg-yolk a day was given at the beginning of the experiment and increased to half a yolk *per diem*, the calcification of bones and teeth was good (Plate III, 5). 138 (Plate III, 5 A), on the basal diet only, developed fairly bad rickets and poorly calcified teeth, whilst 137 (Plate III, 5 B), with additional egg-yolk, appeared to have well calcified teeth and bones. After 8 or 9 weeks, however, the same effect was observed as with the cod-liver oil; the animals having egg-yolk lost weight and died if allowed to remain on this diet (Fig. 5).

Table VIII. *The effect of egg-yolk.*

Age at beginning of experiment: about 10 weeks.
Weight at beginning of experiment: 850–900 g.
Time of year at beginning of experiment: May.
General diet: Oats 4, Bran 1 } 1·5 % $CaCO_3$, 30 g. turnip.

No. of exp.	Variable factor	Time on diet weeks	Gain in weight g.	Calcium in femur shaft: % wet weight	Calcium in femur shaft: Amount g.	X-ray	Histology: Bones	Histology: Teeth
137	¼–½ egg-yolk	11	430	17·3	0·294	Normal	Normal	Calc. good
138	—	11	465	16·1	0·216	Bad rickets	Bad rickets	Calc. bad

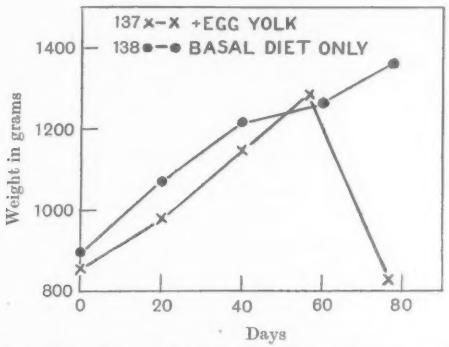

Fig. 5. Weight curves of 137 and 138 (see Plate III, 5). Note rapid loss of weight of rabbit on egg-yolk after 60 days of diet.

In one series of experiments, when cabbage was added to the basal diet as well as egg-yolk, the animal grew better and, when killed at the end of 8½ weeks, was still putting on weight and seemed in excellent health. Photomicrographs of ribs of 250 and 243 (Plate III, 6) show that on the basal diet and cabbage bad rickets resulted (Plate III, 6 B), whereas when egg-yolk was added as well as cabbage calcification was good.

Table IX. *The effect of egg-yolk added to the basal diet and cabbage.*

Age at beginning of experiment: 9½ weeks.
Weight at beginning of experiment: 770–820 g.
Time of year at beginning of experiment: December.
General diet: Crushed oats 4, Bran 1 } 1·5 % $CaCO_3$.
2 g. yeast, 6 cc. neut. lemon juice, 20 g. cabbage.

No. of exp.	Variable factor	Time on diet weeks	Gain in weight g.	Calcium in femur shaft: % wet weight	Calcium in femur shaft: Amount g.	X-ray: Bones	X-ray: Teeth	Histology: Bones	Histology: Teeth
243	¼–1 egg-yolk	8	820	15·3	0·362	Normal	Normal	Normal	Normal
250	—	8	550	13·6	0·224	Rickets	Poor calc.	Rickets	Poor calc.

2. *Vegetable foods.*

Many observations have been made by different workers on the anti-rachitic action of green vegetables in the case of rats and the general consensus of opinion is that their calcifying vitamin content is small and their vitamin A content fairly high. Most of the experiments have been carried out on spinach. Thus McClendon and Shuck [1923] found that 0·1 to 0·5 g. of dried spinach cured ophthalmia but did not protect against rickets in rats, even when it formed 75 % of the diet. Zucker and Barnett [1923] also found that an alcoholic and ether extract of spinach did not protect against rickets in rats but promoted their growth when the diet was otherwise devoid of vitamin A. Similar results were obtained with spinach by Goldblatt and Zilva [1923]. Quite recently the subject has been studied afresh by Chick and Roscoe [1926], who point out that the deficiency of phosphorus in the diets of previous workers on rickets in rats detracted from the reliability of their results. In order to overcome this difficulty Chick and Roscoe raised the phosphorus content of the basal diet to 0·37 %, leaving the calcium at 0·27 %. These investigators found that spinach grown in the open in winter, spring or autumn possessed no demonstrable anti-rachitic properties but that when grown in midsummer it had appreciable anti-rachitic value. Boas [1926] also concluded that winter spinach contained a negligible amount of vitamin D but abundance of A.

E. Mellanby [1925] in his experiments on dogs found that green cabbage had a definite but not powerful anti-rachitic action. When oatmeal was the rickets-producing element of the diet the effect of cabbage was very small, but when white flour was the cereal eaten the anti-rachitic action of the cabbage was more evident. In his experiments and possibly in other experiments on carnivorous animals, one possible source of error in determining the anti-rachitic action of green and other vegetables is the difficulty such animals have in digesting them completely. Often much of the vegetable is passed, apparently undigested and unchanged, in the faeces. Experiments to determine the anti-rachitic properties of grass and vegetables made on rabbits have the merit that these particular kinds of food are normal to the animal and more easily digested.

Since most of the experiments with vegetable foods were carried out in series of animals in litters, so that the effect of one vegetable could be compared with another, the details of some experiments will be given in groups after the main effects of these substances have been briefly described.

(A) *Green foods.*

(i) *Grass.* The addition of grass to the basal rickets-producing diet was followed by varying effects on calcification, the variations depending partly on the amount of grass added and partly on the season of the year during which the grass was grown. This addition always improved the appetite and general health of the animals.

The following are some of the main effects:

(*a*) When grass grown in summer was eaten *ad libitum* in addition to oats 4 parts, bran 1, with and without $CaCO_3$, the animals grew well and calcification was good (Plate III, 7 A).

(*b*) Summer grass to the amount of 50 g. daily sometimes induced normal calcification (Exp. 124, Table XV), but was often accompanied by the development of either slight rickets or a characteristic enlargement of the shafts of radius and ulna (Plate IV, 5 E and Table XIV). This enlargement was associated with a bending of the bones and an eversion of the front paws, and microscopically the bones were abnormal.

(*c*) Summer grass added to the basal diet when limited to 30 g. daily resulted in rickets.

(*d*) Winter-grown grass up to 50 g. daily brought about the development of some rickets but, what was more striking, a condition of scurvy.

(*e*) When the winter grass was increased to 75 g., the long bones of the rabbit were often enlarged and bent (Plate III, 7 B).

It would appear from these results that even small quantities of grass added to the basal diet have some anti-rachitic action, which becomes prominent when fairly large quantities are eaten; also that the potency of this anti-rachitic effect is greater in summer-grown than in winter-grown grass. It is of interest to note that the same difference appears to hold in the anti-scorbutic effect of grass grown at different times of the year, the summer variety being more strongly antiscorbutic than the winter.

(ii) *Cabbage.* If the amount of the basal diet be kept constant, it will usually be found that the addition of cabbage up to about 50 g. makes the rachitic condition worse. This increase in rickets is accompanied by a distinct improvement in the animals' health and in more rapid growth (Fig. 1). From the point of view under which this investigation was started, the effect of adding cabbage to the basal diet is of great interest because it is now possible to keep rabbits for long periods in good health under conditions which allow defective bones and teeth to be formed. Rabbits are still alive which have had the basal diet together with 30–50 g. of cabbage daily for periods up to one year. Such a state of affairs on the basis of the work mentioned in the beginning of this paper might appear to be suitable for the production of dental caries.

The addition of 25 g. of green cabbage leaf daily to 40 g. of the basal diet will result, in the course of a few weeks, in a severer form of rickets and in more defectively calcified teeth than in the animal eating the basal diet only. Doubling the cabbage intake to 50 g. may make the rachitic condition even more severe (Plate IV, 1 A and B). Fifty rabbits receiving, in addition to their basal diet, amounts of cabbage varying from 20 to 50 g. daily, some with and some without lemon juice, developed rickets, in most cases of a severe nature. Plate IV, 1 A and B, represent radiographs of two animals (363 and 362) after $6\frac{1}{2}$ weeks of diet, receiving respectively 30 and 15 g. of cabbage daily. It will

be seen that 363 (30 g. cabbage) has worse rickets than 362 (15 g. cabbage). There was but little difference in the gain in weight of these animals during the $6\frac{1}{2}$ weeks of the diet, 363 gaining 330 g. and 362, 320 g. When the unsaponifiable fraction of 1 cc. of cod-liver oil was eaten in addition to the basal diet and the cabbage, the bones were normally calcified.

Table X. *The effect of adding cabbage to the basal diet.*

Age at beginning of experiment: 6 weeks.
Weight at beginning of experiment: 300–350 g.
Time of year at beginning of experiment: May.
General diet: Rolled oats 4, Bran 1 } 1·5 % $CaCO_3$.
6 cc. neutral lemon juice.

No. of exp.	Variable factor	Time on diet up to radiograph weeks	Gain in weight g.	X-rays
362	Green cabbage leaf, 15 g.	$6\frac{1}{2}$	320	Rickets (Plate IV, 1 B)
363	Green cabbage leaf, 30 g.	$6\frac{1}{2}$	330	Bad rickets (Plate IV, 1 A)
360	Green cabbage leaf, 30 g. Unsaponifiable subs. from 1 cc. cod-liver oil	$6\frac{1}{2}$	345	Normal

If the amount of oats and bran plus $CaCO_3$ in the basal diet be reduced to 25 g. and the cabbage increased to the eating capacity of the rabbit (from 40 to 400 g. according to the size), the calcification processes are greatly improved and the bones and teeth are nearly normal in structure (Plate IV, 2 A, and Fig. 4).

Table XI. *The effect of giving cabbage* ad lib.

Age at beginning of experiment: $7\frac{1}{2}$ weeks.
Weight at beginning of experiment: 500 g. each.
Time of year at beginning of experiment: February.
General diet: Crushed oats 4, Bran 1 } 1·5 % $CaCO_3$.
6 cc. neutral lemon juice.

No. of exp.	Variable factor	Time on diet up to radiograph weeks	Gain in weight g.	X-rays
290	Savoy cabbage *ad lib.* up to 400 g. Oats and bran mixt., average amt. 25 g.	21	875	Very slight rickets (Plate IV, 2 A)
291	Savoy cabbage up to 45 g. Oats and bran mixt. up to 70 g.	21	775	Very bad rickets (Plate IV, 2 B)

Boiling cabbage in water in an open vessel for 8 hours appears to make both the growth and the calcification somewhat worse than with the corresponding amount of uncooked cabbage (Fig. 6 and Plate IV, 3).

No differences in the calcifying powers of cabbage during winter and summer months have been detected nor between the green leaf and the white parts.

These experimental results indicate that the calcifying properties of green cabbage are small compared with the anti-calcifying properties of the basal diet. When the intake is low, *i.e.* below 50 g. daily, it may actually make the

rachitic changes in the bones worse in spite of the improvement in the general health of the rabbits which results.

Table XII. *The effect of boiling cabbage.*

Age at beginning of experiment: 9½ weeks.
Weight at beginning of experiment: 480–510 g.
Time of year at beginning of experiment: September.
General diet: Rolled oats 4, Bran 1 } 1·5 % $CaCO_3$.
6 cc. neutral lemon juice.

No. of exp.	Variable factor	Time on diet months	Gain in weight g.	X-ray	
				10 weeks	10 months
202	Cabbage uncooked, 20–40 g.	10	1200	Rickets (Plate IV, 3 A)	Rickets healing
204	Cabbage cooked in open vessel 8 hours, 20–40 g.	10	750	Worse rickets (Plate IV, 3 B)	Rickets healing very irregularly

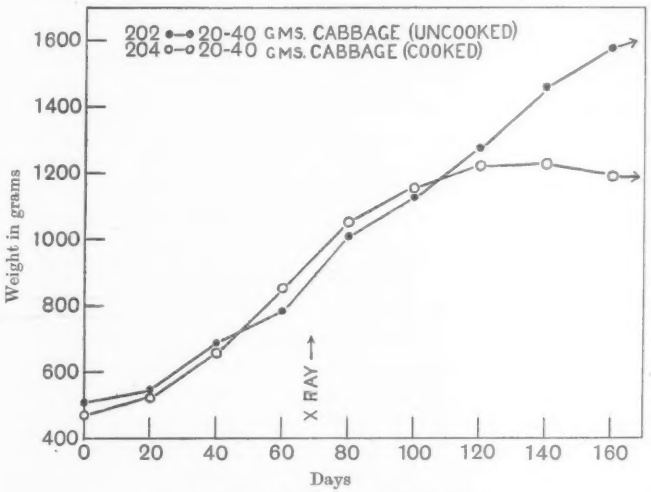

Fig. 6. Weight curves of 202 and 204.

(iii) *Dandelion leaves* were tested with the basal diet in one series during the summer months and resulted in fairly well calcified bones and teeth (Plate IV, 5).

B. *Root vegetables.*

(i) *Turnip.* Animals fed on white turnip up to 100 g. daily in addition to a diet of oats 4 parts, bran 1 part and 1·5 % $CaCO_3$ grew well and developed bad rickets (Plate IV, 4). Growth on white turnip, oats 4, bran 1, without $CaCO_3$, was not nearly so good but rickets developed nevertheless. White turnip seemed to have no anti-rachitic powers but improved the rate of growth and general health of the animals.

(ii) *Swede turnip* gave results depending partly on the quantity eaten and partly on some undetermined factor. Animals given 50 g. swede turnip with the usual basal diet grew well and often showed fairly well calcified, although not normal, bones and teeth (Plate IV, 5 C, and Table XIV).

Animals given 10, 20 and 30 g. swede turnip in addition to the basal diet grew somewhat less rapidly than those with 50 g., but developed rickets.

(iii) *Carrot.* The addition of carrot to the basal diet was also followed by variable results. In eight out of ten experiments calcification was fairly good (Plate IV, 5 D and Table XIV), whilst in two, fed at the same time of the year and with the same amount of carrot, calcification was poor (Table XIII).

Table XIII. *The relative effect of carrot and cabbage.*

Age at beginning of experiment: 10 weeks.
Weight at beginning of experiment: about 800 g.
Time of year at beginning of experiment: May.
General diet: Oats 4 / Bran 1 } 1·5 % $CaCO_3$.
6 cc. neutral lemon juice.

No. of exp.	Variable factor	Time on diet weeks	Gain in weight g.	Calcium % wet weight	X-ray	Histology
118	Carrot to 50 g.	6	735	14·6	Rickets	Rickets
120	Cabbage to 50 g.	6	705	14·0	Rickets	Rickets

(iv) *Potato* has been tested in one case only. The animal was given up to 50 g. potato with the basal diet: it grew fairly well and developed bad rickets (Table XV).

Table XIV. *The relative effect of different vegetables.*

Age at beginning of experiment: 8½ weeks.
Weight at beginning of experiment: 400–600 g.
Time of year at beginning of experiment: July.
General diet: Oats 4 / Bran 1 } 1·5 % $CaCO_3$, *ad lib.*

No. of exp.	Variable factor	Time on diet up to radio-graphs weeks	Gain in weight g.	Calcium in femur shaft: % wet weight	Calcium in femur shaft: Amount g.	X-ray: Radius and ulna at death	X-ray: Jaws and teeth	Histology
169	Dandelion leaves, 20–50 g.	12½	900	16·2	0·346	Nearly normal (more rickets in earlier X-ray) (Plate IV, 5 A)	Nearly normal (Plate V, 4 A)	Nearly normal
170	Cabbage, 20–50 g.	12½	580	14·0	0·136	Bad rickets (Plate IV, 5 B)	Calcification defective (Plate V, 4 B)	Bad rickets
172	Swede turnip, 20–50 g.	12½	550	15·7	0·212	Nearly normal (slight rickets in earlier X-ray) (Plate IV, 5 C)	Nearly normal, but not as well calcified as 169	Calcification fairly good but not normal
173	Carrot, 20–50 g.	12½	770	17·5	0·253	Nearly normal (Plate IV, 5 D)	Nearly normal	Nearly normal
174	Grass, 20–50 g.	12½	900	16·0	0·244	Enlarged ulna (earlier X-ray slight rickets) (Plate IV, 5 E)	Nearly normal	Abnormal

Table XV. *The relative effect of different vegetables.*

Age at beginning of experiment: about 8 weeks.
Weight at beginning of experiment: 450–550 g.
Time of year at beginning of experiment: May.
General diet: Oats 4 / Bran 1 } 1·5 % $CaCO_3$, *ad lib.*

No. of exp.	Variable factor	Time on diet up to radiographs weeks	Gain in weight g.	Calcium in femur shaft, % wet weight	X-ray: Radius and ulna at death	X-ray: Jaws and teeth	Histology of costo-chondral junctions
122	Potato, 20–50 g.	20	380	15·0	Bad rickets	Poorly calcified	Bad rickets
123	Swede turnip, 20–50 g.	20½	860	16·2	Nearly normal	Well calcified	Abnormal—some osteoporosis
124	Grass, 20–50 g.	17	1070	17·2	Nearly normal	Well calcified	Some osteoporosis

Discussion of the effect of vegetables.

It is surprising that the calcifying influence of the vegetables tested is so small, more especially in view of the great improvement in the general health of the animals which follows their addition to the basal diet. Experiments with puppies suggested that those dietetic conditions which promote poor calcification of bones and teeth lead fairly rapidly, *i.e.* after 4 to 6 months, to impaired health, loss of appetite and even death. Experience with rabbits, however, has shown that, for at least a year, when the diet contains cabbage in addition to the basal substances, they may remain in good health in spite of very deformed limbs and defectively calcified teeth. Cabbage, and probably other vegetables, therefore, provide some essential dietetic factors which are absent from or deficient in the basal diets. One of these substances may be vitamin A, but this alone would probably not account for the beneficial effect on the general health. It is clear from the experiments in which normal calcification of bones and teeth resulted when calcifying vitamin was added, either in the form of the unsaponifiable fraction of cod-liver oil or as egg-yolk, that this particular vitamin is only present in small quantities in the vegetables tested.

Of the green foods, grass is apparently more potently anti-rachitic than cabbage, especially when summer-grown. It has also been observed that even the anti-scorbutic action of grass is greater in the summer time. In order to produce perfectly formed bones the intake of green cabbage leaf must be large, and that of the basal substances (*i.e.* oats, bran and $CaCO_3$) small.

White turnip seems to be devoid of anti-rachitic vitamin, but swede turnip is better in this respect, nearly normal calcification of bones being obtained on several occasions when 50 g. were eaten daily. Carrot, on the whole, is more anti-rachitic than other root vegetables but the variable results which were obtained, both with carrot and swede turnip, remain unexplained. The action of the carrot in different series of experiments does not seem to bear any relationship to the season of the year.

EFFECT OF IRRADIATING THE ANIMALS AND THE FOOD.

(*a*) *Animals.* The result of exposing rabbits to a mercury vapour lamp was similar to that obtained by other workers who have irradiated children, rats and dogs. For instance, rabbits 233 and 234, of the same litter, were fed on the basal diet alone and one of them (233) was exposed three times a week to the mercury vapour lamp. The irradiated animal showed normal calcification at the end of 10 weeks, the control developed rickets.

Table XVI. *The effect of ultra-violet radiation on the animal.*

Age at beginning of experiment: 8 weeks.
Weight at beginning of experiment: 450–500 g.
Time of year at beginning of experiment: November.
General diet: Crushed oats 4, Bran 1 } 1·5 % $CaCO_3$.
6 cc. neutral lemon juice.

No. of exp.	Variable factor	Time on diet weeks	Gain in weight g.	X-ray
233	Animal irradiated 3 times a week	10	470	Normal
234	—	10	400	Rickets

Similar results were obtained with animals fed on the basal diet and green cabbage (Plate V, 1 B, Table XVII, Exp. 282).

(*b*) *Foods.* Irradiating the foodstuff also greatly assists calcification and may result in normal or nearly normal bones and teeth. In series 280–286 (see Table XVII), the animals all had the basal diet together with 20–45 g. of cabbage. They grew well (Fig. 7), but the calcification is seen to be nearly normal not only in 282 (Plate V, 1 B), when the animal was irradiated, but also in 280 (Plate V, 1 A) when the basal diet was irradiated, and in 286 (Plate V, 1 D), when the cabbage only was irradiated.

Table XVII. *The effect of irradiating the food.*

Age at beginning of experiment: 7½ weeks.
Weight at beginning of experiment: 310–450 g.
Time of year at beginning of experiment: February.
General diet: Crushed oats 4, Bran 1 } 1·5 % $CaCO_3$.
6 cc. neutral lemon juice, 20–45 g. cabbage.

No. of exp.	Variable factor	Time on diet weeks	X-ray
280	Oats, bran and $CaCO_3$ irradiated	24	Nearly normal
282	Animal irradiated	24	Nearly normal (slightly worse at 10 weeks)
283	—	24	Rickets
286	Cabbage irradiated	24	Nearly normal

See also photomicrographs of the costo-chondral junctions of three rabbits of the same family, 227, 229 and 230 (Plate V, 2), of which 227 had the basal diet only, 229 had the basal diet and irradiated cabbage and 230 received

additional cabbage but without irradiation. It will be seen that 227 (Plate V, 2 A) has rickets, 229 (Plate V, 2 B) is nearly normal, whilst 230 (Plate V, 2 C) has even greater disorganisation of the growing cartilage than 227.

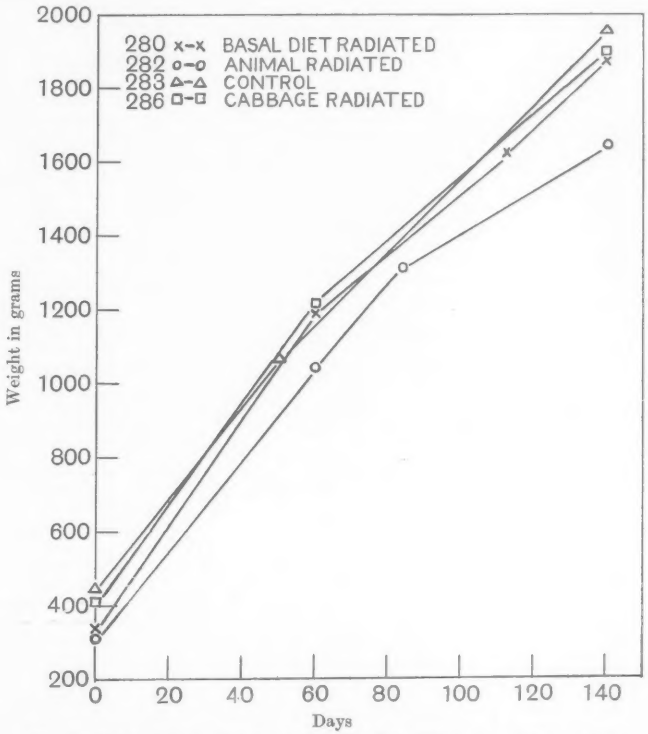

Fig. 7. Weight curves of animals 280, 282, 283, 286, whose radiographs are shown in Plate V, 1. Note the parallel rate of increase in weight.

Summary.

(1) Diets are described which, while allowing good growth and good general health in rabbits, produce very defective calcification of teeth and bones. It is hoped by feeding on these diets to produce ultimately dental caries.

(2) The degree of abnormal calcification is related to the growth of the animal. If x g. of a diet produces rickets, $2x$ g. of the same diet will produce worse rickets.

(3) The worst calcification is obtained in rabbits under these experimental conditions when the calcium and phosphorus content of the diet approaches figures which have been described by workers on rickets in rats as likely to prevent the disease.

(4) Calcification in rabbits responds immediately, in the same way as in other animals studied, to an increase in calcifying vitamin in the diet and to exposure either of the animal or of food to ultra-violet radiations.

(5) Examination of some vegetable foods indicates that they are for the most part deficient in calcifying vitamin. Grass, especially summer-grown grass, contains more than cabbage. Of the root vegetables, carrot and swede turnip contain more than white turnip.

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EXPLANATION OF PLATES.

Plate III, 1. Photographs of (A) normal and (B) rachitic rabbits.

Plate III, 2. Radiographs of forelegs showing the rickets-producing effect of adding 1·5 % $CaCO_3$ to a diet of oats, bran and lemon juice. (A) no $CaCO_3$ (67), (B) $CaCO_3$ (69).

Plate III, 3. Radiograph of rabbit (340) in which Na_2CO_3 replaced $CaCO_3$ in the basal diet. Note the thick band of ossification at epiphysis.

Plate III, 4. Radiograph showing improved calcification on adding cod-liver oil to basal diet.

A. Basal diet + cod-liver oil—good calcification (130).
B. Basal diet only—poor calcification (127).

Plate III, 5. Radiographs showing effect of adding egg-yolk to basal diet.

A (138) received basal diet only—bad rickets.
B (137) received basal diet + egg-yolk—normal calcification.

Plate III, 6. Photomicrographs of costo-chondral junctions showing defective calcification produced by diet which included cabbage, and normal ossification produced by the addition of egg-yolk.

A (243) basal diet + cabbage + egg-yolk—good calcification.
B (250) basal diet + cabbage—very poor calcification and much osteoid tissue.

Plate III, 7. Radiographs showing the effect of grass on calcification.

A (41) summer grass *ad lib.* added to basal diet—good bone formation.
B (61) winter grass (75 g.) added to basal diet. Note great thickening of radius, an effect often produced by grass.

Plate IV, 1. Radiographs showing that rickets may be made worse by adding more cabbage to basal diet.

A (363) basal diet + 30 g. cabbage daily—bad rickets.
B (362) basal diet + 15 g. cabbage daily—moderate rickets.

The rate of growth of these animals was similar.

Plate IV, 2. Radiographs showing the relative effects of cabbage *ad lib.* and cod-liver oil on rickets in rabbits.

A (290) basal diet (25 g.) + savoy cabbage *ad lib.*
B (291) basal diet (70 g.) + 45 g. savoy cabbage.
C (292) basal diet (70 g.) + 45 g. savoy cabbage + unsaponifiable substance from 1 cc. cod-liver oil.
D (294) basal diet (70 g.) + 45 g. savoy cabbage + 0·5 cc. cod-liver oil.

Note that savoy cabbage *ad lib.* (A) has not produced quite the perfect calcification of C and D. B has very bad rickets.

Plate IV, 3. Radiographs showing that boiled cabbage produces worse rickets than uncooked cabbage.

A (202) basal diet + uncooked cabbage—moderate rickets.
B (204) basal diet + cooked cabbage—bad rickets.

Plate IV, 4. Radiograph showing that white turnip up to 100 g. daily added to basal diet allows very bad calcification.

Plate IV, 5. Radiographs showing relative effect on calcification of different vegetables added to basal diet.

A (169) basal diet + dandelion 50 g.—fairly good calcification.
B (170) ,, + cabbage 50 g.—very bad calcification.
C (172) ,, + swede turnip 50 g.—fairly good calcification.
D (173) ,, + carrot 50 g.—fairly good calcification.
E (174) ,, + summer grass 50 g.—thickened bones but little rickets.

Plate V, 1. Radiographs showing effect on calcification of irradiating animal and food with a mercury vapour lamp. All animals on same diet.

A (280) basal diet irradiated—fairly good calcification.
B (282) animal irradiated—fairly good calcification.
C (283) no irradiation—bad calcification.
D (286) cabbage of diet irradiated—good calcification.

Plate V, 2. Photomicrographs of costo-chondral junctions showing effect of cabbage and irradiated cabbage on calcification.

A (227) basal diet only—disordered growth of cartilage.
B (229) basal diet + cabbage (irradiated)—nearly normal growth.
C (230) basal diet + cabbage—disordered growth.

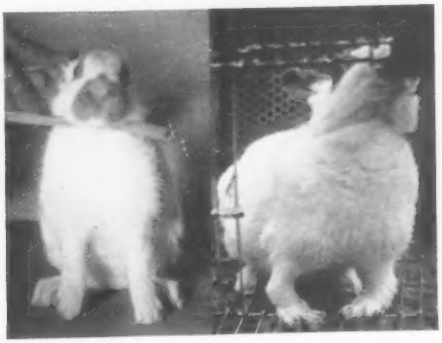
Plate V, 3. Photomicrographs of ground sections of molar teeth of two rabbits showing perfect and imperfect calcification of the dentine.

A. Dentine thick and well calcified.
B. Dentine thin and imperfectly calcified showing interglobular spaces.

Plate V, 4. Radiographs of half lower jaws of two rabbits showing well calcified and poorly calcified bones.

A (169) basal diet + 45 g. dandelion leaf—nearly normal calcification.
B (170) basal diet + 45 g. cabbage leaf—poorly calcified bone easily seen round incisors and growing portion of molars.

A B



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A B

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3

A B

4

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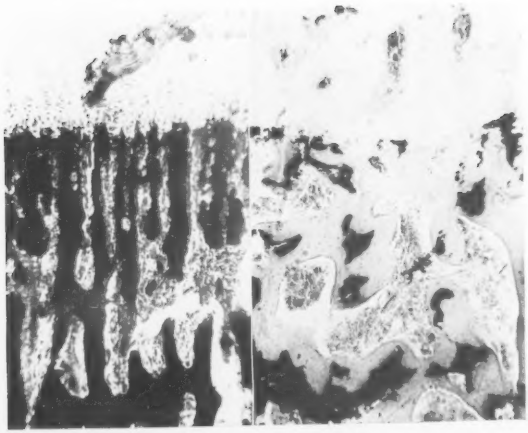

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A B

7

A B

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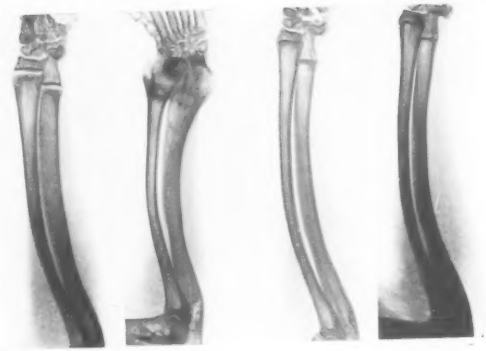
A B C D



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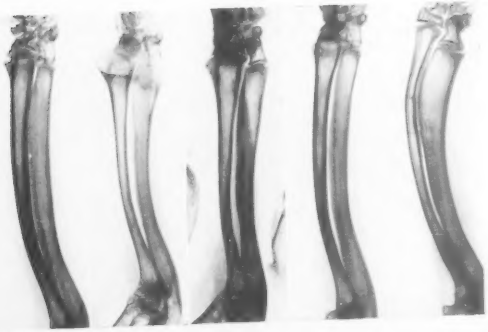
A B

3

4

A B C D E



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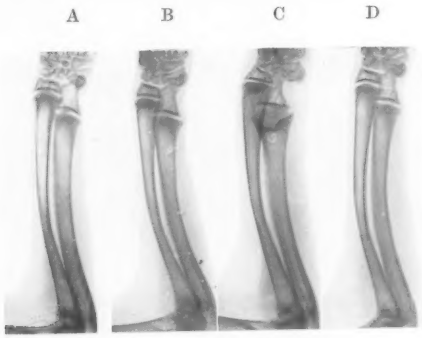

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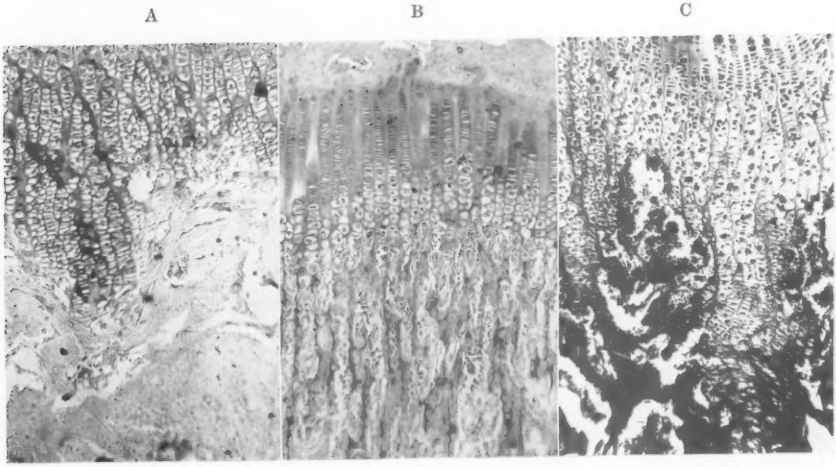

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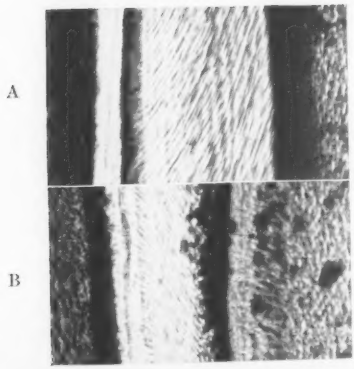

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CXIV. ELECTRODE POTENTIALS OF HERMIDIN, THE CHROMOGEN OF *MERCURIALIS PERENNIS*.

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Haas and Hill [1925, 1] have recently directed attention to a chromogen of considerable interest which escapes from the plasmolysed cells of *Mercurialis perennis*. If the coarsely divided tissue be steeped, anaerobically, in chloroform water for a short time, there results an extract which, on exposure to oxygen, assumes first a rich blue and then a yellow-brown colour. These investigators [1925, 2] have further shown that, within the experimental limits imposed by the difficulty of arresting the oxidation exactly at the stage of maximum intensity of blue colour, the two successive reactions are attended by an equal absorption of oxygen. The oxidation to the blue substance (cyanohermidin) was demonstrated to be readily reversible and Haas and Hill [1926] were further persuaded that the second stage of oxidation is also, at least partially, reversible, although they were unable quantitatively or consistently to effect the reduction of the yellow compound (chrysohermidin) through the blue to the chromogen. The data presented in the papers cited suggested that the systems involved might be amenable to electrometric study and the present paper reports the measure of success attained.

The application of electrometric methods to organic systems has been extensively discussed in its theoretical aspects by Clark and Cohen [1923, 1]. The general relations may be summarised in a form of the Nernst electrode equation

$$E_h = E'_0 - \frac{RT}{nF} \log \frac{[S_r]}{[S_0]}$$

where E_h represents the observed potential compared with that of the normal hydrogen electrode, E_0' is a constant, n is the number of equivalents of hydrogen involved in the reaction and $[S_r]$ and $[S_0]$ are the respective concentrations of the reductant and the oxidant which are in equilibrium at the observed potential. If, by reason of acid-base equilibria, the concentrations of the *actual* oxidant and reductant are not identical with the total concentrations of the reactants, the simple equation must be modified to embrace terms involving the $[H^{\cdot}]$ and the various dissociation constants effective over the range of p_H investigated. E_0' then becomes a function of p_H and the

equilibrium constants. In order to establish fully the equilibrium states in a reversible oxidation-reduction it is, therefore, necessary to determine first the equilibrium potentials at constant [H·] and then to examine the variation of these with p_H.

No attempt has been made to prepare the pigment in the pure state. The orderly character of the results obtained with crude extracts of the plant would seem to indicate that the impurities present were not such as to interfere seriously with the equilibria established at the electrode by hermidin and its oxidation products. When the pure substance becomes available it may be found that there is a displacement of the systems on the oxidation-reduction potential scale, but it is improbable that the main implications of the present work will thereby require modification. On the other hand, the full interpretation of the electrometric data must, perforce, await the elucidation of the structure of the pigment.

Titration curve at constant [H·].

The green shoots or, in some of the work, the colourless rhizomes were cut into small pieces and extracted under nitrogen with enough water to cover them, together with a little ether or chloroform. After an hour the extract was removed, filtered and stored under nitrogen. The nitrogen for this and all operations was purified of all trace of oxygen by passage over heated copper and conducted through copper or glass tubing, rubber connections being avoided. The electrode vessel was of the type employed by Clark and Cohen [1923, 2] carrying two gold-plated platinum electrodes to allow duplicate readings of potential. Contact was made with a saturated calomel electrode by means of a saturated potassium chloride bridge in the usual way and the whole cell was held in a thermostat at $30 \pm 0{\cdot}1°$. 40 cc. of $M/20$ buffer solution (Clark) of the desired p_H were introduced into the vessel and de-aerated with a stream of nitrogen, and 10 cc. of the plant extract were then added from a burette without contact with the air. The gas stream was continued throughout the titration and gave efficient stirring. Preliminary work indicated that the extracts were of the order of $M/100$ with respect to the chromogen and, in consequence, the titrating solutions were made of similar strength. In order that the [H·] should be maintained as constant as possible during the progress of the titration, the titrating solutions were made up by diluting a $M/20$ solution in water with four volumes of the buffer solution to which the plant extract had been added. These solutions were de-aerated before use and held in a protected burette under nitrogen. Experience indicated that, despite all precautions, the hermidin was 5 to 10 % in the oxidised form by the time the extracts had been introduced into the electrode vessel. It was therefore customary, before titrating with an oxidising agent, to make a preliminary titration with sodium hydrosulphite until the electrode indicated complete reduction. This permitted the complete curve to be traced by the subsequent oxidation.

The system hermidin-cyanohermidin.

Studies of the first stage in the oxidation of hermidin gave very satisfactory results. On any one plant extract the curves traced by titrating the chromogen with benzoquinone, potassium ferricyanide or oxygen, or by reducing the blue compound with sodium hydrosulphite were, within the experimental error, identical.

Independent electrodes came rapidly into agreement at each step in the titration and the equilibrium potentials were remarkably constant. With extracts prepared on different days the value of E_0' was found to vary, the whole curve being correspondingly displaced. In the worst case this discrepancy reached a value of 0·01v. For the explanation of this disconcerting fact we may have to await the preparation of the pure pigment.

The curve in Fig. 1 has been constructed from data abbreviated in Table I and represents a typical titration curve. Having made a graphic approximation of the end-point, the value of E_0' is disclosed since this is equal to the potential at the mid-point of the curve, and it remains to calculate n and thence the theoretical potentials corresponding to the experimental values of $\frac{[S_r]}{[S_0]}$. The electrode equation may be written in the form

$$n = \frac{0{\cdot}0601}{E'_0 - E_h} \log \frac{[S_r]}{[S_0]}$$

in which the value of RT/F at 30° has been substituted. In Table II this equation has been solved for a number of observed potentials on the flat portion of the curve. Since n is a whole number it is obvious that its value is 2. This reaction, in common with the majority of reversible organic oxidation-reductions is thus seen to be one involving the loss or gain of an electron pair, *i.e.* two equivalents of hydrogen.

A comparison of the observed potentials with those calculated from the electrode equation is made in Table I. It will be seen that the deviations exceed a few tenths of a millivolt only towards the end of the titration where we have reason to suspect interference in the equilibria due to the production of chrysohermidin (the yellow oxidation product).

Table I. *Titration of hermidin with M/150 benzoquinone.*

p_H 3·99 Temp. 30° E_0' +0·1266

Quinone cc.	Percentage oxidation	E_h volts found	E_h volts calculated	Deviation volts
0·80	8·25	+0·0956	+0·0952	+0·0004
1·40	14·43	0·1030	0·1034	−0·0004
2·40	24·74	0·1121	0·1121	—
3·40	35·05	0·1187	0·1186	+0·0001
4·40	45·36	0·1241	0·1242	−0·0001
5·40	55·67	0·1298	0·1296	+0·0002
6·90	71·12	0·1386	0·1384	+0·0002
7·90	81·43	0·1459	0·1459	—
8·90	91·75	0·1571	0·1580	−0·0009
9·10	93·80	0·1585	0·1610	−0·0025
9·47	97·61	0·1656	0·1750	−0·0094
9·70 (end-point)				

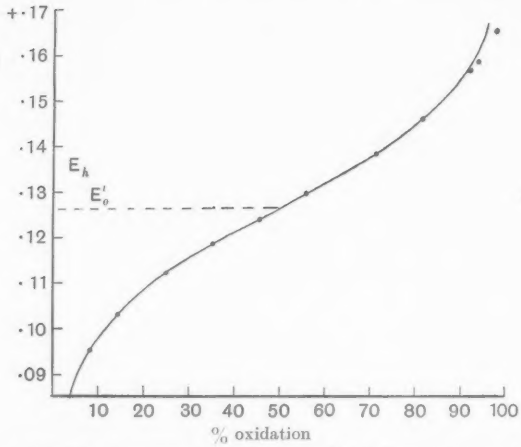

Fig. 1 (cf. Table I). Titration of hermidin with $M/150$ benzoquinone at p_H 3·99.

Table II (cf. Table I). *The value of* n.

$E_0' - E_h$	Percentage oxidation	$0{\cdot}0601 \log \frac{[S_r]}{[S_0]}$	n
+0·0236	14·43	+0·0465	1·97
+0·0079	35·05	+0·0161	2·04
+0·0025	45·36	+0·0049	1·94
−0·0032	55·67	−0·0059	1·86
−0·0120	71·12	−0·0235	1·96
−0·0305	91·75	−0·0629	2·06
		Mean	1·97

The relation of E_0' to p_H.

This relation was investigated by determining the equilibrium potentials of 5 cc. of a mixture of hermidin and cyanohermidin—approximately equimolecular—added to 20 cc. of a series of $M/20$ buffer solutions with the usual precautions against intrusion of oxygen. One of the buffer solutions chosen was that employed in determining the titration curve. In this way a constant correction was derived to bring the potential of the mixture, for which the exact value of the ratio $\frac{[S_r]}{[S_0]}$ was unknown, to that of a mixture whose ratio was unity. This is, of course, the value E_0'. Owing to the unknown buffering power of the plant extract the p_H of the buffer-pigment mixture might differ appreciably from that of a buffer diluted with an amount of water equal to the volume of extract used. To overcome this difficulty advantage was taken of the irreversibility of the oxidation to chrysohermidin in acid solution (see later). A sample of the extract (which has an acid reaction) was allowed to oxidise in the air and was warmed to complete the formation of chrysohermidin. 5 cc. of this solution were added to 20 cc. of each buffer solution and the [H·] determined by the hydrogen electrode. The results are assembled in Table III and Fig. 2. The reader is again reminded that the value of E_0'

at a given [H·] was reproducible only with a single sample of plant extract. For this reason the actual values reported may be taken to be accurate only for the material actually used in the experiments quoted. The data of Tables I–IV refer to a single extract.

Table III. *Hermidin-cyanohermidin. Relation of E_0' to p_H.*

The calculated values of E_0' have been derived from the equation

$$E_0' = E_0 + 0{\cdot}03006 \log [K_2 (H^+)^2 + (H^+)^3] - 0{\cdot}03006 \log [K_0 + (H^+)].$$

E_0 has been given the value $+0{\cdot}3665$ and the hypothetical constants K_0 and K_2 the values $K_0 = 1{\cdot}0 \times 10^{-8}$ and $K_2 = 5{\cdot}0 \times 10^{-7}$.

		E_0'		
p_H	Buffer	found	calc.	Deviation
2·0	Phthalate	+0·2460	+0·2462	−0·0002
2·45	,,	0·2185	0·2192	−0·0007
3·08	,,	0·1817	0·1814	+0·0003
3·47	,,	0·1579	0·1579	—
3·99	,,	0·1266	0·1266	—
4·54	,,	0·0931	0·0939	−0·0008
5·30	,,	0·0490	0·0490	—
5·64	Phosphate	0·0295	0·0298	−0·0003
6·08	,,	+0·0071	+0·0067	+0·0004
6·57	,,	−0·0156	−0·0153	−0·0003
6·89	,,	−0·0286	−0·0279	−0·0007
7·33	,,	−0·0446	−0·0445	−0·0001
7·61	,,	−0·0555	−0·0555	—
7·91	,,	−0·0679	−0·0681	+0·0002
8·16	Borate	−0·0788	−0·0797	+0·0009
8·86	,,	−0·1158	−0·1169	+0·0011

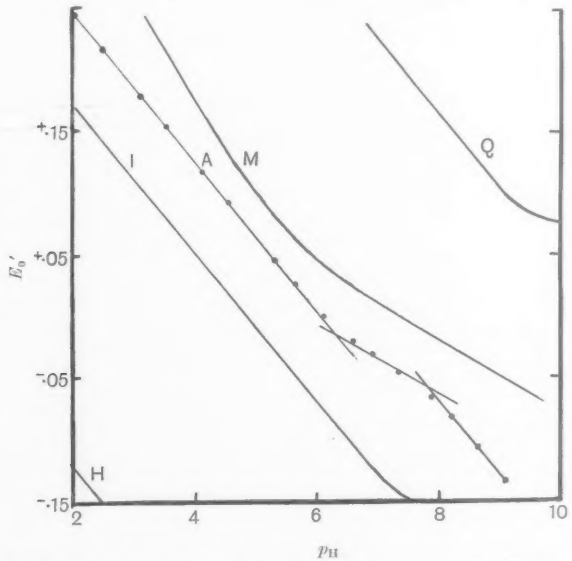

Fig. 2 (cf. Table III). E_0' : p_H curves. Potentials of 50 % mixtures of oxidant and reductant of hermidin and certain other systems [Clark, 1925] at various [H·]. *A*, hermidin-cyanohermidin; *I*, indigo white—indigo carmine; *M*, methylene white—methylene blue; *Q*, quinhydrone; *H*, hydrogen electrode.

The interpretation of the E_0' : p_H curve is seriously hampered by our ignorance of the structures participating in the equilibria. From p_H 2 to about 6 the slope of the curve indicates an increasing negative potential of 0·06v. per unit of p_H. The curve here changes towards a "0·03 slope" but reverts, about p_H 8, to the original direction. Probably the values in the alkaline range are uncertain as borate buffers alone were available and these have been found to give anomalous results in other cases. Alkaline to p_H 9 the potentials became subject to uncertain drifts which made precarious the continuation of the curve beyond p_H 9. The intersections of the two slopes are indicative of acid-base dissociations at the corresponding [H·], but nothing can be said of their nature. A variety of electrode equations may be derived, involving a variety of equilibrium constants, and would equally well satisfy the observed results. One has been chosen in Table III solely to provide a means of indicating the general symmetry of the results, and it is not intended that the assumptions upon which the equation is based should be taken to indicate correctly the acid-base character of the molecule. It is assumed that the inflexion in the more acid region is due to the destruction of an anion by the oxidation (K_2). The second inflexion is attributed to an acid group common to both oxidant and reductant but differing in strength in the two reactants. In the oxidant its constant is K_0 whilst in the reductant it is too weak to have influence within the experimental range of [H·]. These assumptions lead to the equation in Table III. The agreement between observed and calculated values of E_0' is within the accuracy of the [H·] determinations.

The system hermidin-cyanohermidin-chrysohermidin.

When the titration was continued beyond the end-point indicated in Fig. 1, a further region of poised potentials was encountered corresponding to the second stage in the oxidation of hermidin. In general, however, no equilibrium potentials could be determined as persistent and rapid drifts were found. However, in the region of p_H 7 to 8 the drifts were slow and it was possible to trace very satisfactory curves by reading the potential as soon as the electrode had made its initial rapid adjustment to each addition of titrating agent and so pursuing the titration as rapidly as possible. The lower curve in Fig. 3 and the data of Table IV record such a titration.

The complete curve should be the resultant of the curves given by

$$E_h = E_{0'_1} + 0{\cdot}03006 \log \frac{\alpha_1}{1-\alpha_1} = E_{0'_2} + 0{\cdot}03006 \log \frac{\alpha_2}{1-\alpha_2}$$

where $_1$ and $_2$ refer respectively to the first and the second stages in the oxidation and α is the fraction of the system which is present in the oxidised form. Having made a graphic approximation to the values of the end-point of the whole reaction and to $E_{0'_1}$ and $E_{0'_2}$, the equations may be solved for α_1 and α_2. The sum $100\left(\frac{\alpha_1+\alpha_2}{2}\right)$ is the percentage oxidation at any point in the titration and this value may be compared with that given by the titre and the end-

point. In Table IV the values of $E_{0'_1}$ and $E_{0'_2}$ have been subjected to minor adjustment to improve the agreement between the equations and the observed results.

Table IV (cf. Fig. 3). *Titration of hermidin with M/100 benzoquinone at* p_H 7·61.

First stage of oxidation $E_{0'_1} = -0{\cdot}0557$
Second ,, $E_{0'_2} = +0{\cdot}0158$

Quinone cc.	E_h volts	α_1	α_2	Percentage total oxidation: calc.	found	Deviation
0·70	−0·0814	0·1225	0·0006	6·16	6·08	−0·08
1·00	0·0763	0·1712	0·0009	8·61	8·69	+0·08
1·40	0·0704	0·2448	0·0014	12·31	12·17	−0·14
1·80	0·0659	0·3140	0·0019	15·80	15·45	−0·35
2·40	0·0600	0·4183	0·0030	21·07	20·86	−0·21
3·00	0·0548	0·5171	0·0043	26·07	26·08	+0·01
4·20	0·0442	0·7068	0·0099	35·84	36·52	+0·68
4·60	0·0392	0·7798	0·0145	39·72	40·00	+0·28
5·10	0·0330	0·8506	0·0233	43·70	44·35	+0·65
5·45	0·0263	0·9047	0·0382	47·15	47·39	+0·24
5·65	0·0220	0·9299	0·0525	49·12	49·13	+0·01
5·90	0·0138	0·9620	0·0939	52·80	51·30	−1·50
6·30	0·0092	0·973	0·1285	55·08	54·78	−0·30
6·80	−0·0019	0·984	0·2046	59·43	59·13	−0·30
7·30	+0·0040	0·989	0·2883	63·85	63·48	−0·37
7·90	0·0092	0·993	0·3763	68·46	68·70	+0·24
8·60	0·0159	0·996	0·4904	74·89	74·78	−0·11
9·10	0·0197	0·998	0·5741	78·60	79·13	+0·53
9·55	0·0239	0·999	0·6504	82·47	83·04	+0·57
10·40	0·0339	1·000	0·8000	90·00	90·43	+0·43
11·05	0·0480	1·000	0·9212	96·06	96·10	+0·04
11·20	0·0515	1·000	0·9380	96·90	97·40	+0·50
11·50 (end-point)						

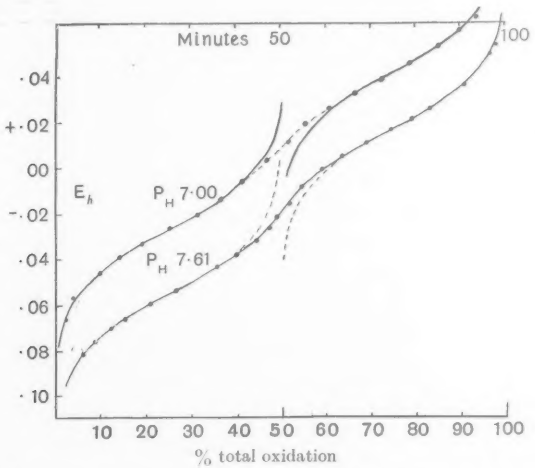

Fig. 3 (cf. Table IV). Lower curve. Titration curve of hermidin with *M*/100 benzoquinone at p_H 7·61. Upper curve. Change of potential with time in hermidin solution buffered at p_H 7·00 when submitted to stream of oxygen diluted with nitrogen to 2 %.

The drifts in potentials corresponding to the second stage in the oxidation of hermidin became accelerated with increasing [H$^{\cdot}$]. From a survey of the electrode behaviour of this system and the fact that these drifts were consistently towards more negative values such as would result from the progressive increase in the ratio $\frac{[S_r]}{[S_0]}$ by loss of oxidant in a secondary irreversible change, the suggestion is made tentatively that the true oxidant of cyanohermidin is subject to such an irreversible change whose velocity is a function of [H$^{\cdot}$]. This change does not appear to be oxidative, but there is nothing further to indicate its character. The series of reactions may, therefore, be summarised in the form

$$\underset{\text{(colourless)}}{\text{Hermidin}} \overset{2H}{\rightleftharpoons} \underset{\text{(blue)}}{\text{Cyanohermidin}} \overset{2H}{\rightleftharpoons} \text{Oxidant} \longrightarrow \underset{\text{(yellow)}}{\text{Chrysohermidin}}$$

The curve expressing the relation of the ratio of the reactants in a reversible oxidation-reduction to the electrode potential has been compared with an acid-base titration curve. In hermidin under restricted conditions of [H$^{\cdot}$] we have the first recorded example of an oxidation-reduction comparable with a system exhibiting two acid-base dissociations.

The rate of oxidation by molecular oxygen.

The writer has made a number of observations on the changes in potential of an inert electrode in solutions of the leuco-compounds of electromotively active dyes—*e.g.* methylene blue, phenol-indophenols—when submitted to a controlled stream of oxygen. If the potentials observed be plotted against time from the initiation of the stream of oxygen there results a curve which, over the significant portion of its course, is a replica of the titration curve at the same p_{H}. The possibility of the electrode behaving as an oxygen electrode does not, therefore, appear to arise and the conclusion must be that, under the particular experimental conditions, the rate of oxidation of the reduced dye is constant throughout the reaction. If this be general, the method offers a simple form of exploratory titration under conditions—*e.g.* presence of oxidisable impurities—where the employment of a more "active" oxidising agent would give uncertain results. The method is, however, limited to those systems and to those ranges of [H$^{\cdot}$] within which the rate of oxidation of the given compound proceeds at a measurable rate. If the rate be too slow the procedure is inconvenient, and if too rapid the inherent lag in the response of the electrode to the constantly changing equilibrium becomes a serious factor which, moreover, will be reflected unevenly at different stages in the reaction. Results by the oxygen method must, however, be suspect unless controlled by other more conventional methods. In the present case this control is not wanting and it would seem, therefore, that the ordered character of the deductions respecting the rates of oxidation is significant.

The upper curve in Fig. 3 represents the results obtained in an experiment

differing from the quinone titration only in that oxidation was effected by a rapid stream of nitrogen containing about 2 % of oxygen. The curve makes sufficiently evident what a detailed analysis of the data fully confirms, that the amount of reductant oxidised per minute is constant throughout the reaction. In Table V are summarised a series of such "oxygen titrations." The first point of interest is that the rates of oxidation of the two stages of oxidation are almost identical in every case. Now the experimental conditions are highly limited and this result might be held to have no general significance had it not been found that other pigments studied under identical conditions were oxidised at widely differing rates. It is suggested that the results observed indicate that the two stages in the oxidation involve very similar chemical groups. In the second place it seems from a comparison of the rates of oxidation at different [H·] that the former vary inversely as the square root of the [H·].

Table V. *Effect of* [$H^{\cdot}$] *on the rate of oxidation of hermidin and of cyanohermidin by a stream of oxygen diluted with nitrogen to* 2 %.

From the oxygen "titration curve" the value of the end-point in minutes and of $E_0'_1$ and $E_0'_2$ were determined graphically. With these the theoretical curves were superimposed upon the composite curve.

		Span of curve		Rate % per min.		$k = v\sqrt{[H^+]}$	
p_H	c_H	1	2	1	2	1	2
6·59	$2{\cdot}55 \times 10^{-8}$	80 min.	75 min.	1·25 %	1·33 %	$6{\cdot}3 \times 10^{-4}$	$6{\cdot}7 \times 10^{-4}$
7·00	$1{\cdot}0 \times 10^{-8}$	51 ,,	49 ,,	1·96	2·04	$6{\cdot}2 \times 10^{-4}$	$6{\cdot}5 \times 10^{-4}$
7·42	$0{\cdot}38 \times 10^{-8}$	30 ,,	29 ,,	3·33	3·45	$6{\cdot}5 \times 10^{-4}$	$6{\cdot}7 \times 10^{-4}$
7·81	$0{\cdot}16 \times 10^{-8}$	20 ,,	20 ,,	5·00	5·00	$6{\cdot}2 \times 10^{-4}$	$6{\cdot}2 \times 10^{-4}$

Discussion.

Haas and Hill have suggested that hermidin may properly be classed with the respiratory pigments of Palladin. The present paper makes no immediate contribution to this question but rather provides more of the quantitative data upon which must be made the final assessment of the respiratory rôle of hermidin. One thing is clear. The reversible oxidation of the chromogen is not a change involving a specific dissociable compound with oxygen but is an oxidation-reduction to be formulated in electrochemical terms. Cyanohermidin is a hydrogen acceptor of the same type as methylene blue and quinone. Were methylene white to be found as a natural constituent of a living cell we should find it difficult to refuse to it a function in respiration. We must not, however, be over-persuaded by the traditional importance to tissue respiration which an experimental convenience has conferred upon this dye, since, apart from glutathione—and the electrode behaviour of this system is not typical—no reversible system of the type here considered has yet been shown to have a fundamental rôle in the respiration of any cell. Yet the lack of evidence is no denial, for it can scarcely be doubted that, under conditions which are not improbable within the living cell, hermidin must function not

only as an efficient hydrogen "transferrer" but, as its ease of oxidation by molecular oxygen implies, as an active oxygen carrier.

From the present work it may be concluded that hermidin exists within the plant in the reduced form to the extent of not less than 95 %. *Mercurialis* is an example of a plant with an oxidation-reduction indicator occurring naturally within it. There is no question, therefore, of the imposition of artificial conditions by the injection of an alien dye. Now the reduction potential of hermidin which is 95 % reduced corresponds with a reduction intensity represented by r_H 10. By the micro-injection of indicators Needham and Needham [1925, 1926] have found that the majority of unicellular organisms which have been studied have given a reduction intensity corresponding to a system in equilibrium with a hypothetical pressure of hydrogen of 10^{-18} atmospheres (*i.e.* a r_H of 18). Only organisms having pronounced anaerobic characteristics, and these only in the absence of oxygen, have shown reduction intensities approaching r_H 10. We hesitate to classify *Mercurialis* with the facultative anaerobes. Moreover, since *Mercurialis* is a green plant there remains to be explained the occurrence within the same cell of a photosynthetic production of oxygen under the pressure of the atmosphere and a state of reduction of hermidin which, it may be calculated, can be in *equilibrium* with a pressure of oxygen of only 10^{-60} atmospheres. It is obvious that the cell sap containing the pigment is not in simple equilibrium with the chloroplast. The theoretical potential of the oxygen electrode is 1·23v. and that of the hermidin electrode 0·35v. more positive than that of the hydrogen electrode: so that a potential difference of almost a volt would thereby exist within the cell. This extravagant figure is mitigated by the fact that oxygen does not appear to exert anything like its full theoretical potential in biological systems. The direct observations of Cannan, Cohen and Clark [1926] and the micro-injection studies referred to agree in giving to "biological oxygen" an oxidation intensity of about r_H 18 corresponding to a potential about 0·54v. positive to that of the hydrogen electrode. This would still leave a potential difference of 0·2v. within the cell containing reduced hermidin. The importance of such an intracellular gradient of reduction intensity cannot be denied, but our knowledge of the conditions pertaining in protoplasm is far too scanty to permit this point to be laboured at present. The determination of the r_H of the whole cell, like the determination of the p_H, may well provide results of much interest but, at the best, would seem to be a precarious undertaking. Protoplasm is a polyphasic system and we shall not expect homogeneous equilibria to prevail.

Summary.

1. The electrode potentials of the system hermidin-cyanohermidin have been determined over the range p_H 2·0 to 8·0 and of the system cyanohermidin-oxidation product over the range p_H 7·0 to 8·0.

2. It is suggested that the second system is reversible only in the neigh-

bourhood of neutrality because the true oxidant is subject to an irreversible non-oxidative change whose velocity is a function of the [H$^{\cdot}$].

3. Some electrometric studies of the rates of oxidation of hermidin and cyanohermidin in relation to [H$^{\cdot}$] are recorded.

4. The significance of these results to the suggested respiratory rôle of hermidin, and to the question of oxidation-reduction equilibria within the cell, is discussed.

The writer gratefully acknowledges the valuable criticism of Prof. W. M. Clark and the continued interest of Dr P. Haas and Dr T. G. Hill, to whom, further, he is indebted for the supply of *Mercurialis*.

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CXV. HAEMOCYANIN.

PART II. THE INFLUENCE OF HYDROGEN ION CONCENTRATION ON THE DISSOCIATION CURVE OF THE OXYHAEMOCYANIN FROM THE BLOOD OF THE COMMON LOBSTER (*HOMARUS VULGARIS*)

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In Part I of this series the authors [1925] examined the blood of four species of decapod crustacea with the object of ascertaining whether the haemocyanins contained therein were capable of exercising respiratory functions. In so far as their power of combining reversibly with oxygen was concerned, the results left no doubt that the pigments were able so to function. An examination of the dissociation curves revealed two further points of interest. Firstly, they proved to be identical, thus suggesting the identity of the haemocyanins in the four species. Secondly, they departed from the hyperbolic form which would be expected if the combination with oxygen followed the law of mass action as formulated for true solutions, and were, in fact, of an S-shape similar to the characteristic oxygen dissociation curve of haemoglobin. It was obvious that, apart from the isolation and chemical investigation of the pigments, the question of their identity could only be settled with any degree of certainty by an examination of their dissociation curves under strictly comparable conditions of hydrogen ion and salt concentrations. An investigation of the effect of changes in these two variables was, moreover, necessary if attempts were to be made to elucidate the cause of the inapplicability of the mass-law in its simple form. The latter problem is of special interest in view of the analogous problem in the case of haemoglobin. Both pigments, or, to be strictly accurate, both groups of pigments, belong to the class of proteins into the elementary composition of which a metal enters. Further, as implied by their classification as proteins, the physical and physico-chemical properties of their solutions present certain common features. It is therefore probable, especially in view of the similarity between the forms of their oxygen dissociation curves, that the factors controlling or modifying their affinities for oxygen are fundamentally of the same nature. What has hitherto been regarded as the special problem of haemoglobin thus becomes a more general one, and it is doubtful if any solution of it can be considered

satisfactory unless it satisfies the data derived from studies of both series of pigments. On the other hand, the more extensive and varied the material with which a particular phenomenon may be studied the greater are the possibilities for elucidating its cause; it is thus conceivable that advances in our knowledge concerning the factors modifying the affinity of haemocyanin for oxygen may contribute to an explanation of the inflexion in the oxygen dissociation curve of haemoglobin.

These considerations have led the authors to investigate the oxygen dissociation curves of various haemocyanins in dialysed solution and to study the effect thereon of changes in hydrogen ion concentration and of the addition of salts. The present communication deals mainly with haemocyanin from the common lobster, and is confined to the influence of hydrogen ion concentration. A few observations relating to the pigments from other species are also included.

Experimental methods.

Preparation of solutions of haemocyanin. The blood, obtained in the manner previously described, was allowed to flow on to solid sodium fluoride (about 1 g. for each 100 cc. of blood) and was stirred vigorously for some minutes to prevent the formation of a gel. After standing for a few hours in the ice-chest, the clot was removed by filtration and the serum concentrated in collodion ultra-filters. The latter process appeared advisable, not only because of the small oxygen capacity of the serum, but in order to compensate for the dilution which occurred in the subsequent process of dialysis. The concentrated serum was then dialysed in collodion membranes against cold water saturated with chloroform until free from salts, filtered from small amounts of protein which had separated, and repeatedly evacuated to remove traces of dissolved chloroform. Adjustments of the hydrogen ion concentration, where necessary, were effected by the addition of a solution of potassium hydroxide.

Determinations of p_H. These were made, unless otherwise stated, electrometrically using a Hildebrand electrode. The measurements were carried out at room temperature, which varied in different experiments from 12° to 20°; no correction was made for deviations of the hydrogen pressure from normal. Before each determination the hydrogen electrode was tested with $M/20$ potassium hydrogen phthalate. The value obtained for the p_H of this solution never deviated by more than 0·02 from the value of 3·97 given by Clark for this buffer solution between the temperatures of 20° and 30°; usually the deviation was not greater than 0·01. As a check against any gross error, the p_H was also, in each case, determined colorimetrically, using standards prepared at intervals of p_H 0·2 as recommended by Clark. For the latter estimations, the haemocyanin solutions were diluted to 1 in 10 with distilled water, and the comparison with the standards carried out in a comparator in the customary manner. Values in satisfactory agreement with those determined electrometrically were obtained over those ranges in which it was possible to use

cresol red or phenol red as indicator. With bromothymol blue and thymol blue, however, the results were less satisfactory. This accords with the experience of other workers, who have also found that the protein error of these indicators is large.

Equilibration of solutions with oxygen at different pressures. More refined methods than those described in Part I have been employed. About 8 cc. of the haemocyanin solution were drawn into a tonometer, which was then swept out and filled under slight pressure with a mixture of nitrogen and oxygen prepared in the proportions necessary to give approximately the desired oxygen pressure. The tonometers were placed in a thermostat and rotated until they attained the temperature of the latter, for which purpose from 15 to 30 minutes were allowed. One stopcock of the tonometer was then caused to project above the surface of the water and opened momentarily in order to equalise the pressure of the contained gases with that of the atmosphere. Rotation in the thermostat was then continued for a further period of from 30 to 45 minutes. Any variations which occurred in the temperature of the thermostat were so small that they were not detectable with a thermometer graduated in tenths of a degree. The exact composition of the gas mixtures in equilibrium with the haemocyanin solutions was determined by analyses of samples of the gases obtained directly from the tonometers; a Haldane portable gas analysis apparatus was employed for this purpose.

Determination of oxygen content of solutions. Two small modifications have been made in the method previously described.

The mixture of hydrosulphite and anthraquinone-β-sulphonate recommended by Fieser [1924] has been used as absorbent for oxygen in place of the hydrosulphite solution described by Van Slyke. Apart from the superior absorbing powers claimed for it by Fieser, the former mixture possesses the advantage that the decomposition of the hydrosulphite, as judged by the formation of sulphuretted hydrogen, proceeds more slowly in the presence of the sulphonic acid than in its absence.

For the extraction of the gases from the equilibrated haemocyanin solutions, 5 cc. samples were introduced into the Van Slyke apparatus in the manner previously described and shaken mechanically for a period of 5 minutes. This extension of the period of extraction, although not necessary with many of the solutions employed, was deemed advisable with certain samples which exhibited a relatively high oxygen capacity at low oxygen pressures, and was accordingly adopted uniformly throughout the experiments.

As evidence for the applicability of the technique employed, it was pointed out in connection with the authors' original experiments that extraction of the serum in the Van Slyke apparatus caused the complete disappearance of the blue colour due to the oxyhaemocyanin. A simple calculation will show that the error due to incomplete extraction is, in most cases, negligible. The volume per cent., v, of oxygen obtained from the sample under examination is, if one omits the factor correcting for the oxygen dissolved at the pressure

existing in the pipette of the apparatus, given by the equation [Van Slyke and Neill, 1924]

$$v = P \frac{0{\cdot}1316a}{(\text{cc. sample})} \times \frac{1}{1+0{\cdot}00384t}$$

where P is the partial pressure of the oxygen at the volume a, and t is the temperature. In the experiments recorded in this communication 5 cc. samples of solution were employed; hence the volume of oxygen, measured at N.T.P., extracted from the sample is $v/20$. Since the total volume of liquid introduced into the pipette is 6 cc. (5 cc. solution + 1 cc. water), and the level of the mercury during extraction of the sample is at a point approximately 2 cc. above the 50 cc. mark of the pipette, the pressure of oxygen in the apparatus on the completion of extraction is, neglecting the small temperature correction, $\frac{v}{20} \times \frac{760}{42}$. When $v = 4$, which is about the maximum value obtained in these experiments, the oxygen pressure in the pipette is thus of the order of 3·6 mm.; for most experiments it is considerably less and frequently does not exceed a fraction of a mm. Hence, on withdrawal from the pipette, the samples are in equilibrium with oxygen pressures varying from 0·3 to 3·6 mm.; that the amount of oxygen thereby lost is, in most cases, negligible is evident from an inspection of the dissociation curves. In cases where it would cause an appreciable, though small, error an approximate correction can be obtained graphically from a large scale curve.

Results.

Dissociation curves of oxyhaemocyanin from Homarus. The data obtained in five series of experiments, all of which were carried out at a temperature of 23°, are given in Table I.

The first three columns (p_H, O_2-pressure and O_2-content) contain the experimentally determined values and require no further comment. In column 4 are shown the corrections which must be added, on account of incomplete extraction by the apparatus, to the figures in column 3 to obtain the true oxygen content of the respective samples. These corrections have been determined graphically in the manner described above and can consequently only be regarded as approximately correct; nevertheless, any error should fall well within the experimental error of the methods employed. In those cases where the corrections would be very small they have been neglected entirely. The values for the physically dissolved oxygen (column 5) have been calculated on the basis that the solubility of oxygen in dialysed haemocyanin solutions is the same as in pure water. For this purpose, it has been assumed, taking the mean of values appearing in tables of physical constants, that water contains 0·62 volumes per cent. of oxygen when in equilibrium with an oxygen pressure of 159 mm. and at a temperature of 23°. The figures in column 6 have been obtained by subtracting the corresponding figure in column 5 from the sum of those in columns 3 and 4. Finally, column 7

Table I

p_H	Oxygen pressure (mm.)	Oxygen content (vols. %)	Correction for oxygen retained	Physically dissolved oxygen (vols. %)	Combined oxygen (vols. %)	Percentage saturation
9·65	17·8	0·422	0·010	0·069	0·363	33·0
	27·2	0·544	0·020	0·106	0·458	42·1
	65·1	0·889	0·030	0·253	0·666	60·6
	98·9	1·102	0·040	0·385	0·757	68·8
	152·7	1·378	0·045	0·594	0·829	75·3
	337·8	2·214	0·080	1·320	0·974	88·5
	619·4	3·338	0·100	2·410	1·028	93·5
	650·0	3·477	0·100	2·530	1·047	95·2
					1·100	100·0
9·50	12·9	0·281	—	0·040	0·241	17·2
	26·6	0·512	—	0·085	0·427	34·7
	32·4	0·624	—	0·105	0·519	37·5
	71·8	1·043	0·010	0·240	0·813	58·1
	154·2	1·710	0·020	0·525	1·205	86·1
	190·9	1·806	0·040	0·660	1·185	84·7
	513·2	3·166	0·065	1·800	1·431	102·2
	556·3	3·270	0·070	1·955	1·385	99·0
	654·6	3·608	0·080	2·300	1·388	99·1
					1·400	100·0
8·75	11·6	0·190	—	0·045	0·145	8·5
	20·7	0·313	—	0·085	0·228	13·4
	38·3	1·008	—	0·155	0·853	50·0
	61·0	1·578	—	0·245	1·333	78·2
	151·6	2·165	0·020	0·575	1·610	94·4
	452·3	3·380	0·040	1·720	1·700	99·7
	552·2	3·755	0·045	2·090	1·710	100·3
	655·3	4·135	0·050	2·480	1·705	100·0
					1·705	100·0
8·26	42·8	0·397	—	0·165	0·232	18·6
	58·2	0·571	—	0·225	0·346	27·7
	125·7	1·359	—	0·490	0·869	69·5
	154·9	1·639	—	0·605	1·034	82·8
	258·8	2·262	—	1·020	1·242	99·3
	487·2	3·168	—	1·920	1·248	99·8
	599·7	3·597	—	2·365	1·232	98·6
	696·7	4·036	—	2·750	1·286	102·8
					1·250	100·0
7·76	61·0	0·459	—	0·230	0·229	14·3
	99·9	0·694	—	0·390	0·304	19·0
	153·2	1·054	—	0·605	0·449	28·1
	230·5	1·610	—	0·915	0·695	43·4
	271·7	1·983	—	1·080	0·903	56·4
	542·8	3·630	—	2·160	1·470	91·9
	556·2	3·697	—	2·215	1·482	92·6
	694·1	4·368	—	2·780	1·588	99·2
					1·600	100·0

shows the percentage saturation of the haemocyanin for each oxygen pressure. This has been calculated from the preceding column, using the figures given on the last line of each series of experiments as representing the maximum amount of oxygen with which the haemocyanin is capable of combining. From the data in Table I five dissociation curves have been plotted and are shown in Fig. 1.

Before discussing these curves, some explanation is necessary respecting, firstly, the selection of the above-mentioned value for the physically dissolved oxygen and, secondly, the determination of the saturation value for the various

haemocyanin solutions. It was anticipated, when the experiments were commenced, that correction for physically dissolved oxygen could be made, as was done in the first paper of this series, from the upper portion of a curve in which oxygen content was plotted against oxygen pressure. Proportionality between these magnitudes did, in fact, occur in the region of high pressures, and the solubility curves deduced therefrom were found to be in fairly satisfactory agreement with the value of 0·62 for 159 mm., thus indicating that the haemocyanin had no influence on the solubility of oxygen. It therefore seemed more accurate to employ the theoretical figure of 0·62 throughout the experiments than to use the actual solubility curves determined in each individual experiment, especially as observed departures from this value were usually in the direction of a slightly greater solubility. Whilst high values could be attributed to experimental error, another explanation is more probable,

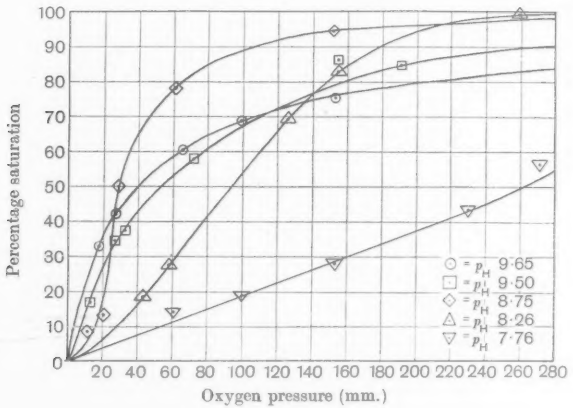

Fig. 1.

namely, the incomplete saturation of the haemocyanin even at the high pressures employed. That this is the case in the experiments at p_H 9·65 is evident from an inspection of Fig. 2, in which oxygen content (curve *A*) and combined oxygen (curve *B*) have been plotted against oxygen pressure, curve *B* being obtained from curve *A* on the above assumption that the solution dissolves oxygen to the same extent as pure water. This particular curve has been chosen partly because it represents the extreme case encountered and partly because of its importance. It further illustrates the difficulty of determining the saturation value. Saturation has obviously not been reached at the highest pressure employed and it has therefore been necessary to select a value somewhat arbitrarily. As can be seen from Table I, this has been estimated as 1·1 vol. per cent. In the remaining experiments the corresponding curves indicated that saturation was reached at lower pressures, and hence less difficulty was experienced in this respect.

The general influence of p_H on the dissociation curves can be seen from Fig. 1. Within the limits (p_H 9·65 to 7·76) examined in these experiments an increase in alkalinity produces two effects: it causes the curve to rise more steeply from the origin and to approach saturation less abruptly. As a consequence of these two effects the various curves intersect one another, and it may, in general, be assumed that a given curve will cross, in succession, every curve lying on its acid side. There will, no doubt, be a limit to this process, but its determination must await further experimental work.

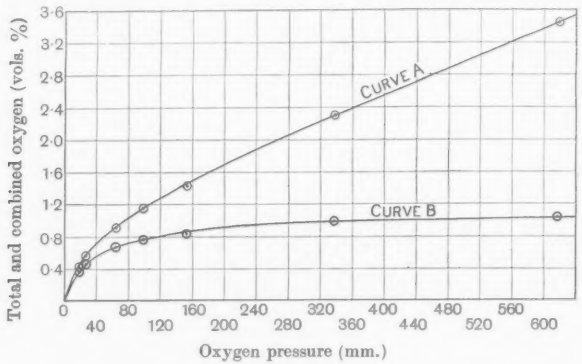

Fig. 2.

Since this intersection of the dissociation curves is probably of fundamental importance for any theory of the causes underlying the inflexion of the curves and has not, as far as the authors are aware, been observed in the case of haemoglobin, it appeared desirable to obtain further evidence of the phenomenon. The following experiment was therefore carried out. Equal volumes of a dialysed haemocyanin solution were brought to different H-ion concentrations by the addition of varying amounts of 0·1087 *N* potassium hydroxide, the final volumes being made up to 20 cc. by the addition of distilled water. The p_H and oxygen content, when equilibrated with air (oxygen pressure 153 mm.), of each solution were then determined. The experimental figures will be found in Table II.

Table II.

Vol. of dialysed haemocyanin sol. (cc.)	15	15	15	15	15	15	15
Vol. of KOH (cc.)	0	0·5	1·5	2·1	2·7	3·3	3·9
Distilled water to (cc.)	20	20	20	20	20	20	20
p_H	7·59	7·84	8·33	8·52	8·79	9·02	9·31
Oxygen content (vols. %)	0·801	0·951	1·806	1·850	1·807	1·589	1·450
Combined oxygen (vols. %)	0·204	0·353	1·209	1·253	1·210	0·992	0·853

The results have been plotted in Fig. 3 which shows the relation between p_H and the amount of oxygen bound by equal quantities of haemocyanin, at the same concentration, when at a temperature of 23° and in equilibrium with an oxygen pressure of 153 mm. The curve shows a maximum at a p_H of about

8·60, which is in good agreement with the positions of the curves in Fig. 1; since there is no doubt that the dissociation curves of the more alkaline haemocyanin solutions are initially steeper than the remainder, this confirms the intersection of the curves. The maximum shown in Fig. 3 naturally holds only for one oxygen pressure, namely, 153 mm. With lower oxygen pressures it would obviously be displaced towards the left, *i.e.* would occur at lower H-ion concentrations, whilst with higher pressures displacement would be to the right.

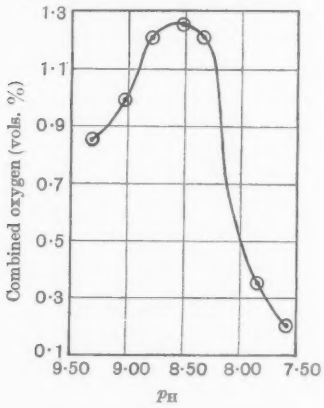

Fig. 3.

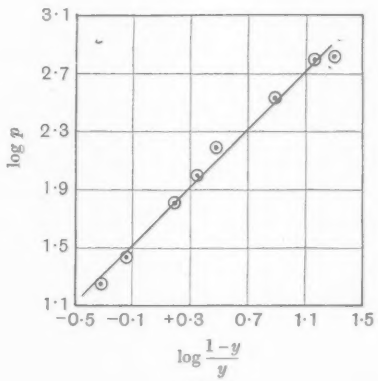

Fig. 4.

The result of the two changes occurring in the shape of the dissociation curve on increasing the alkalinity of the solution is to cause the S-shape to become less pronounced. At p_H 9·65, as an inspection of Fig. 1 will show, it is no longer apparent; moreover, as far as can be determined by the methods available, the curve approaches saturation asymptotically, as is evident from curve *B* in Fig. 2. At this p_H the curve is, at any rate superficially, of the hyperbolic type which would be expected if the dissociation of oxyhaemocyanin were a unimolecular reaction obeying the law of mass action. If it be assumed that this is in fact the case and that the reaction proceeds according to the equation

$$HcO_2 \rightleftharpoons Hc + O_2 \qquad \text{.....(1)}$$

then

$$\frac{[HcO_2]}{[Hc][O_2]} = K \qquad \text{.....(2),}$$

where the square brackets denote concentrations and K is the equilibrium constant of the reaction. If, further, $[Hc] = y$, then $[HcO_2] = 1 - y$; moreover, assuming the validity of Henry's law, $[O_2] \propto p$, where p is the partial pressure of oxygen in equilibrium with the solution, equation 2 becomes

$$\frac{1-y}{y} = kp.$$

Taking logarithms,

$$\log \frac{1-y}{y} = \log k + \log p.$$

Hence, if $\log (1 - y)/y$ is plotted against $\log p$ a straight line inclined at an angle of 45° to the abscissa should be obtained if equation (1) correctly represents the reaction in question. Under other conditions the slope should be different. Such a curve has been drawn for the results obtained at p_H 9·65 and is reproduced in Fig. 4. The points fall satisfactorily about a straight line inclined to the axis at an angle of 45°.

In view of the relatively high p_H at which this result was obtained the suggestion arose that the disappearance of the point of inflexion and the appearance of a curve, which, if not a hyperbola, at least approximates to one, coincide with the complete conversion of the haemocyanin into its potassium salt. It had been found by the following experiment that the isoelectric point of haemocyanin (*Homarus*) is in the neighbourhood of p_H 4·8. A series of acetate-acetic acid buffers with the same concentration of sodium acetate were prepared and to 9 cc. of each buffer solution 1 cc. of dialysed serum was added. The calculated p_H of the solutions are shown below, while the formation and extent of any turbidity produced on addition of the dialysed serum are denoted by crosses beneath these figures.

p_H	4·2	4·4	4·6	4·8	5·0	5·2	5·4
	+	+	+ + + +	+ + + + +	+ + +	+ +	+ +

The value obtained from this experiment may not accurately represent the isoelectric point, but it is sufficient to show that in the dissociation curves in Fig. 1 the haemocyanin is well on the alkaline side of the isoelectric point and to support the view that at p_H 9·65 the pigment is entirely in the form of its alkali salt. To obtain further evidence on the latter point, 10 cc. of a dialysed haemocyanin solution were titrated with 0·1087 *N* KOH, the p_H of the solution being followed electrometrically during the course of the titration. The results of this electrometric titration are presented graphically in Fig. 5. The

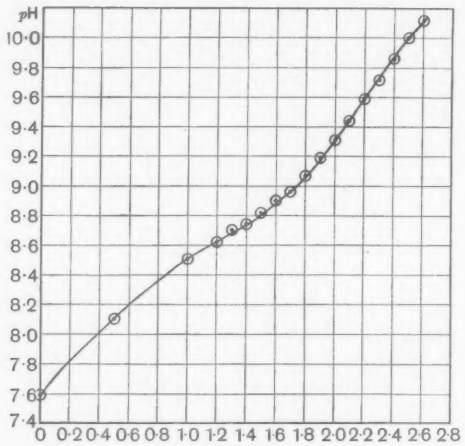

Fig. 5.

curve resembles that of the titration curve of a weak acid and, from its course, it is clear that from about p_H 9·0 onwards practically the whole of the haemocyanin is present in the form of its alkali salt.

Comparison of haemocyanins from different species.

A few preliminary observations are recorded.

Limulus. In view of the similarity between the dissociation curves of the haemocyanin in the serum of *Limulus* and of those in other species (see Part I), the former pigment has been examined. The dialysed or undialysed serum differs so markedly in external characteristics from the corresponding sera of *Cancer, Maia, Palinurus,* or *Homarus,* that further proof that the pigments are different seems almost unnecessary. *Limulus* serum presents a turbid appearance; its colour is pale green by transmitted light and a relatively light blue by reflected light. When dialysed, the turbidity disappears but the colours undergo little change. According to the precipitation method described above in connection with haemocyanin from *Homarus*, the isoelectric point of *Limulus* haemocyanin is not less than 6·3. At a p_H of about 6·8 and in the absence of salts its affinity for oxygen at a temperature of 9° is so great that the methods employed by the authors for the determination of the oxygen capacity of *Homarus* serum are no longer applicable without modification. Under these conditions, the haemocyanin is roughly 80 % saturated at an oxygen pressure of 2 mm. Addition of acid or alkali to the dialysed serum in sufficient amount to bring the p_H to 5·5 or 8·0 respectively does not appear to produce any marked change in the steepness of the dissociation curve. Hence no conclusion can be drawn as to the shape of the initial portions of the curves. For this reason, and because the above p_H values were only determined colorimetrically and are therefore probably not very accurate, the authors are refraining from publishing the actual curves obtained. The above information is sufficient to indicate that *Limulus* haemocyanin differs profoundly from *Homarus* haemocyanin.

Homarus, Cancer, Maia, and Palinurus. The sera from these animals when free from pink pigments, resemble one another closely. As seen by reflected light they appear to be a deep rich blue in colour; by transmitted light they are practically colourless or faintly green. During the process of dialysis the colour by transmitted light is enormously intensified, becoming a deep green or bluish-green; simultaneously, the blue colour by reflected light undergoes considerable diminution. This immediately suggests that the reflected blue colour of the original serum is due to the Tyndall phenomenon, a suggestion which appears to be confirmed by the fact that addition of salt or acid, but not of alkali, to the dialysed serum practically restores its original appearance. As shown above, the isoelectric point of *Homarus* haemocyanin is at about p_H 4·8; using the same method, a similar value was obtained for *Cancer* haemocyanin. The approximate isoelectric points of the pigments from *Maia* and *Palinurus* have not been determined. Despite this resemblance between

the haemocyanins from these four species and the identity of the dissociation curves determined on the undialysed sera, work, which is not yet complete, on the dialysed serum of *Cancer* has given results which conclusively show that *Cancer* haemocyanin is different from *Homarus* haemocyanin. It is therefore probable that the pigments from all four species are different, although they resemble one another in many ways.

Summary.

The work recorded above has shown that, between p_H 7·8 and 9·65, the shape of the dissociation curve of *Homarus* haemocyanin is modified in two directions by changes in H-ion concentration. Increase in alkalinity causes the initial portion of the curve to become steeper while the upper portion simultaneously tends to approach saturation more and more asymptotically. At p_H 9·65 the effect of these two processes is seen at its maximum; as a result, the inflexion of the curve, as far as can be determined by the methods available, disappears while the curve itself is indistinguishable from a rectangular hyperbola. Applying methods similar to those which have been employed in connection with haemoglobin it has been further shown that the behaviour of haemocyanin at p_H 9·65 is consonant with the view that the combination with oxygen follows the law of mass action as formulated for true solutions. It may be concluded from the evidence presented that at this p_H the pigment is present entirely in the form of its alkali salt; hence it would appear that at lower p_H the inflexion in the curve is in some way connected with the fact that the haemocyanin is present, not in one form (*i.e.* as alkali salt or anion), but as a mixture of different forms (*e.g.* anion and undissociated molecule). Further experimental work which, it is hoped, will contribute to the elucidation of this point, is being carried out.

Limulus haemocyanin is different from that of *Cancer*, *Homarus*, *Maia*, and *Palinurus*. The pigments from the last four species resemble one another closely in properties but are probably not identical; in the case of *Cancer* and *Homarus* they are certainly different.

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CXVI. HAEMOCYANIN.

PART III. THE INFLUENCE OF HYDROGEN ION CONCENTRATION ON THE DISSOCIATION CURVE OF THE OXYHAEMOCYANIN FROM THE BLOOD OF THE EDIBLE CRAB (*CANCER PAGURUS*).

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The experiments on the effect of p_H on the oxygen dissociation curve of the haemocyanin from *Homarus* described in the preceding paper have now been extended to the pigment from *Cancer*.

Experimental methods.

The technique employed was essentially the same as that used in Part II and need not, therefore, be described in detail. The following modifications or additions have, however, been introduced.

Preparation of solutions of haemocyanin. The experiments on *Homarus* were confined to $[H^{\cdot}]$ on the alkaline side of the isoelectric point, the more acid solutions of the pigment being obtained by dialysing the serum for a sufficient length of time against distilled water. In the present series of experiments on the haemocyanin from *Cancer*, it was desired to extend the observations to solutions on the acid side of the isoelectric point as well as to those which approached this point more closely on the alkaline side. As previously recorded, the isoelectric point of haemocyanin from *Cancer* is, according to the method of optimum precipitation, at about p_H 4·8. To approach or pass through this point, addition of acid is obviously necessary. Trial experiments showed, however, that the precipitate of haemocyanin formed by addition of acid could not be re-dissolved without such dilution of the solution as to render it useless for the experiments. The following method, which proved extremely effective, was therefore adopted. The concentrated serum was dialysed in closed collodion membranes against distilled water until free from salts. The distilled water was then replaced by an acid solution of suitable strength. With very dilute acids the p_H of the dialysed serum was reduced without the occurrence of precipitation. With more concentrated acids, the haemocyanin was precipitated as a coherent mass which filled the collodion membrane.

By allowing dialysis against the acid to proceed, this mass slowly re-dissolved and a clear solution was finally obtained. Excessive dilution of the solution and denaturation of the protein were thus obviated; further, any salts which might be formed during the process would be removed by dialysis.

In studying the effect of p_H on the dissociation curves, it has been the authors' aim in the present experiments, as well as in those of Part II, to work with solutions as free from salt as possible. In conformity with this aim, the addition of salts (*e.g.* in the form of buffer solutions) to the dialysed serum has been avoided; when adjustments in p_H have been necessary after removal of the solutions from the collodion sacs, these have, in general, been made by the addition of alkali when the solution was on the alkaline side of the isoelectric point and of acid when on the acid side. In only one instance has this procedure been departed from, namely, in the case of the curve at p_H 5·60 (see Fig. 2). The solution employed for the series of estimations represented by this curve was brought from about p_H 6·0 to 5·60 by the addition of a small amount of acid, although the solution was on the alkaline side of the isoelectric point. It is thought, however, that any salts which may possibly have been formed in this process would be so small in amount that any influence which they might exert on the shape of the dissociation curve would be negligible.

Determination of the oxygen content of solutions. The dissociation curves of haemocyanin from *Cancer* on the extreme acid and alkaline sides of the isoelectric point are so steep that the Van Slyke technique as hitherto employed by the authors is inapplicable without modification to facilitate the extraction of the oxygen. For information leading to a solution of this problem the authors are indebted to Mr A. C. Redfield, who informed them in a private communication that potassium cyanide completely displaces the oxygen from the oxyhaemocyanin from *Limulus*. This reagent also acts similarly towards oxyhaemocyanin from *Cancer*, but unfortunately it cannot be employed for the determination of those curves where the use of such a reagent is essential owing to the fact that its solution is so strongly alkaline that it would cause the precipitation of the protein in the pipette of the apparatus. On the assumption that the cyanogen ions were responsible for the displacement of the oxygen, it appeared that another cyanide would be equally efficient. The only soluble cyanide available which does not suffer from the same disadvantages as potassium cyanide is, however, mercuric cyanide, and this is practically unionised. Nevertheless, it was thought that solutions of this salt might contain sufficient cyanogen ions to displace the oxygen from the oxyhaemocyanin, while the concentration of mercuric ions would be so small as to be without effect on the protein. Some tests which were carried out showed that the amount of oxygen extracted from alkaline haemocyanin solutions was, in fact, greater in the presence than in the absence of mercuric cyanide. That mercuric cyanide is less efficient than either potassium cyanide or hydrocyanic acid in this respect is, however, shown by the fact that whereas the two latter reagents completely decolorise haemocyanin when in contact with air, mer-

curic cyanide does not do so although it causes a considerable diminution in the intensity of the colour. These results suggest that one is dealing with two reversible reactions in which cyanogen ions and oxygen are competing for the haemocyanin. If this is so, reduction in the concentration of the oxygen would favour the combination of the haemocyanin with the cyanogen ions and, at the extremely low oxygen pressures existing in the pipette of the Van Slyke apparatus, one would expect the combination with the cyanogen ions to be complete. On the assumption that these views are correct, mercuric cyanide has been employed to facilitate the extraction of the oxygen in the following way. About 7 cc. of a saturated solution of mercuric cyanide are introduced into the Van Slyke apparatus, completely extracted, and displaced into the cup of the apparatus. The sample of haemocyanin to be extracted is then introduced into the pipette and washed in with 3 cc. of the mercuric cyanide solution. The remainder of the estimation is then carried out in the manner previously described. In order to assist the action of the cyanide reagent to the greatest possible degree, the oxygen pressure in the apparatus has been reduced to the minimum consistent with accuracy by varying the volume of the sample according to the oxygen pressure with which it had been equilibrated. Thus, with low oxygen contents 5 cc. samples were employed, while 3 cc. and 2 cc. samples were used with solutions of medium and high oxygen content respectively. Although it has been demonstrated that the mercuric cyanide reagent does facilitate the extraction of the oxygen, the real justification for its use lies in the fact that the values for physically dissolved oxygen obtained from the different series of experiments are all in approximate agreement with the value for pure water.

In calculating the volumes per cent. of oxygen contained in the haemocyanin solutions the complete equation, including the solubility factor, of Van Slyke and Neill [1924] has been employed; the use of the mercuric cyanide has rendered unnecessary and inapplicable the method of graphical correction outlined in Part II.

Results.

Table I contains the data derived from eight series of experiments on haemocyanin solutions from *Cancer*. All samples were equilibrated with the gas mixtures at a temperature of 23°.

Table I.

p_H	Oxygen pressure (mm.)	Oxygen content (vols. %)	Physically dissolved oxygen (vols. %)	Combined oxygen (vols. %)	Percentage saturation
9·52	3·82	0·292	0·015	0·277	14·4
	7·38	0·610	0·029	0·581	30·2
	13·47	1·104	0·053	1·051	54·7
	22·11	1·421	0·086	1·335	69·6
	79·99	1·958	0·312	1·646	85·7
	151·75	2·378	0·592	1·786	93·0
	256·44	2·864	1·000	1·864	97·2
	375·83	3·375	1·466	1·909	99·4
				1·920	100·0

Table I (*continued*)

p_H	Oxygen pressure (mm.)	Oxygen content (vols. %)	Physically dissolved oxygen (vols. %)	Combined oxygen (vols. %)	Percentage saturation
9·25	4·05	0·315	0·016	0·299	12·2
	7·74	0·801	0·030	0·771	32·8
	23·29	1·999	0·091	1·908	81·2
	89·35	2·536	0·348	2·188	89·5
	155·94	2·840	0·607	2·233	95·0
	276·32	3·424	1·078	2·346	100·0
	364·41	3·786	1·421	2·365	100·7
	476·68	4·207	1·859	2·348	100·0
				2·347	100·0
9·17	4·37	0·153	0·017	0·136	6·8
	7·78	0·417	0·030	0·387	19·3
	15·17	1·394	0·059	1·335	66·7
	17·08	1·580	0·067	1·513	75·6
	27·27	1·908	0·106	1·817	90·8
	153·64	2·542	0·599	1·968	98·4
	432·62	3·626	1·687	1·984	99·2
	699·06	4·655	2·726	2·009	100·4
				2·000	100·0
7·35	64·99	0·545	0·253	0·292	13·3
	99·16	1·066	0·387	0·679	31·0
	152·38	1·623	0·594	1·029	46·8
	230·82	2·732	0·900	1·832	83·6
	256·56	2·896	1·001	1·895	86·4
	435·49	3·889	1·698	2·191	99·8
	599·18	4·531	2·337	2·194	100·0
				2·194	100·0
6·21	33·69	0·467	0·131	0·336	16·1
	69·95	0·995	0·273	0·722	34·7
	89·55	1·309	0·349	0·960	46·2
	153·85	2·224	0·600	1·624	78·1
	257·78	2·866	1·005	1·861	89·5
	519·60	4·025	2·026	1·999	96·1
	591·46	4·418	2·307	2·111	101·5
	715·75	4·864	2·791	2·073	99·6
				2·080	100·0
5·60	12·09	0·347	0·047	0·300	17·4
	31·81	0·960	0·124	0·836	48·5
	56·22	1·467	0·219	1·248	72·3
	126·08	2·089	0·492	1·597	92·5
	153·64	2·213	0·599	1·614	93·6
	280·37	2·817	1·093	1·724	99·9
	384·31	3·205	1·479	1·726	100·0
				1·726	100·0
4·19	4·03	0·242	0·016	0·226	11·3
	8·49	0·583	0·033	0·550	27·5
	10·17	0·701	0·040	0·661	33·0
	35·91	1·692	0·140	1·552	77·6
	82·96	2·067	0·324	1·743	87·1
	122·06	2·429	0·476	1·953	97·6
	155·52	2·514	0·607	1·907	95·3
	242·26	2·928	0·945	1·983	99·1
	337·46	3·356	1·316	2·040	102·0
	469·17	3·787	1·830	1·957	97·8
				2·000	100·0
3·84	3·01	0·195	0·012	0·183	24·1
	7·81	0·403	0·030	0·373	49·1
	12·72	0·549	0·050	0·499	65·7
	55·53	0·830	0·217	0·613	80·7
	119·22	1·167	0·465	0·702	92·4
	151·54	1·333	0·591	0·742	97·6
	252·04	1·733	0·983	0·750	98·7
				0·760	100·0

The columns in Table I correspond with those in Table I of Part II except that, for reasons stated above, it has been unnecessary to introduce any correction to be added to the experimentally determined values. It should be stated, however, that in the experiments carried out at p_H 9·17 no mercuric cyanide was employed. In this case a small graphical correction has, therefore, been made and incorporated in the figures representing oxygen content. The physically dissolved oxygen has again been calculated on the assumption that at an oxygen pressure of 159 mm. and a temperature of 23° the haemocyanin solutions dissolve 0·62 volumes per cent. of oxygen. From the data in Table I eight dissociation curves have been plotted and are reproduced in Figs. 1 and 2.

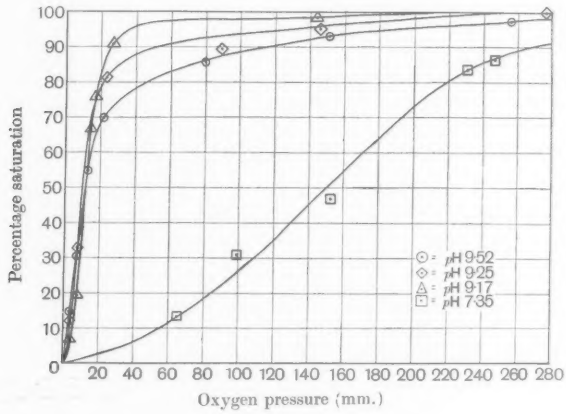

Fig. 1.

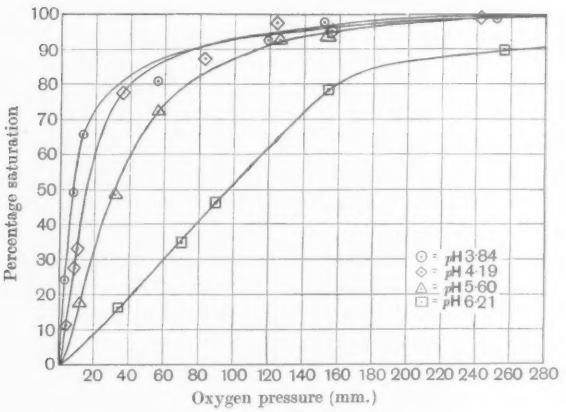

Fig. 2.

Discussion.

The four dissociation curves obtained with the more alkaline solutions and shown in Fig 1 cover a p_H range approximating to that investigated in the case of *Homarus*. A comparison of these curves with those in Fig. 1 of Part II of this series indicates that the general influence of p_H on the dissociation curves of the pigments from the two species is the same. The intersection of the curves commented upon in connection with haemocyanin from *Homarus* can again be observed in Fig. 1, indicating that the more alkaline the solution the more does the curve approximate to the hyperbola which would be expected if the combination of the haemocyanin with oxygen followed the laws applicable to homogeneous solutions. The most alkaline solution (p_H 9·52) investigated, however, is not quite of the hyperbolic type, and an attempt to determine the curve for a more alkaline solution (p_H 9·95) failed owing to the haemocyanin slowly undergoing gelation. Incidentally, it should be noted that even in the form of a gel the pigment was still able to combine reversibly with oxygen. Despite this failure to realise the conditions necessary to give a hyperbolic curve, it seems clear from the general trend of the curves in Fig. 1 that if one could increase the alkalinity of the solution above p_H 9·52 and still retain the haemocyanin in the form of a sol such a curve would finally be obtained.

One further point emerges from a comparison of the curves from *Cancer* with those from *Homarus*. Although changes in p_H produce the same general effect, it is clear that the affinity of *Cancer* haemocyanin for oxygen as expressed by the steepness of the dissociation curves, is, within the p_H range investigated, greater than that of *Homarus* haemocyanin at the same p_H. This forms the experimental proof for the statement made in Part II that the pigments from the two species are not identical.

The curves obtained with the more acid solutions are shown in Fig. 2. It is at once evident that they form the counterpart of the curves in Fig. 1. The intersection again appears although, probably owing to the greater p_H interval between the successive curves, not in such a pronounced form. Just as increase in alkalinity causes the curves in Fig. 1 to approximate more and more closely to the hyperbolic form so, in Fig. 2, increase in acidity produces the same effect. The points from which the curve at p_H 3·84 has been drawn, when tested by the method employed in Part II, appear, within the limits of experimental error, to correspond with the combination with oxygen following the law of mass action. Unfortunately, the oxygen content of the solution employed in this series of experiments was relatively low, suggesting that during the addition of acid some of the pigment was converted into a form which no longer yielded oxygen to a vacuum. At a somewhat greater acidity the haemocyanin loses its capacity for combining reversibly with oxygen although it retains a light green colour.

From the foregoing it is obvious that the affinity of haemocyanin for

oxygen passes through a minimum at a certain p_H. This minimum appears, from the experiments here recorded, to occur in the neighbourhood of the neutral point. That such a minimum exists has also been recently shown by Hogben [1926] using a colorimetric method based upon that of Redfield and Hurd [1925]. Its significance is not, however, clear. Hogben appears to reject the view that it corresponds with the isoelectric point and to prefer the explanation that it is due to the pigment existing in tautomeric forms. The experiments described above, as well as those in Part II, suggest that the shape of the dissociation curve is controlled, in some manner as yet undetermined, by the relative amounts of pigment present in the form of salt (either with acid or base) and in the uncombined form. The greater the proportion of salt the greater is the affinity of the haemocyanin for oxygen and the more nearly does the dissociation curve assume a hyperbolic form. From this behaviour a minimum affinity for oxygen would be expected at the isoelectric point. The chief difficulty in the way of this interpretation is the fact that the isoelectric point, assuming that it coincides with optimum precipitation, appears to be at about p_H 4·8, while the minimum affinity occurs at about p_H 7. According to Michaelis [1922], however, the point of optimum precipitation only coincides with the isoelectric point provided that any salt formed remains completely ionised. This question cannot, therefore, be decided until the isoelectric point has been determined by another method.

There is one further point to which attention should be directed. It is clear both from the experiments on *Cancer* and *Homarus* haemocyanin that the pigment is, under the conditions employed, by no means completely saturated with oxygen until oxygen pressures considerably greater than that due to the atmosphere are reached. Thus, at p_H 7·35 and a temperature of 23° *Cancer* haemocyanin is only approximately 50 % saturated when in equilibrium with an oxygen partial pressure corresponding with that in the atmosphere. This result is not without significance for methods of determining dissociation curves based upon colorimetry. Although the colorimetric method might be expected to give accurate results and possesses the advantage of being rapid and simple, it is evident that curves obtained by this method under different conditions of acidity will not be comparable unless the standards are, in each case, maintained at the same degree of saturation. This condition will not be fulfilled in the case of the haemocyanin from *Cancer* and *Homarus* if the standards are in equilibrium with air and the temperature is as high as 23°. It is improbable that they were fulfilled at the slightly lower temperature of 18·7° employed by Hogben in the case of *Cancer* serum, unless, indeed, the salts added in the form of buffer mixtures as well as those present in the serum itself profoundly modified the shape of the curves. Whilst the colorimetric method is undoubtedly capable of indicating the general influence of p_H on a particular haemocyanin, the results so obtained can have no quantitative significance unless care is taken to avoid the sources of error indicated above.

SUMMARY.

The oxygen dissociation curves of haemocyanin from *Cancer* have been determined at p_H values ranging from 3·84 to 9·52. The affinity of the haemocyanin for oxygen, as expressed by the steepness of the dissociation curve, is at a minimum in the neighbourhood of neutrality. As the acidity or alkalinity of the solution is increased the curve approaches more and more closely to the hyperbolic form which would be expected if the combination with oxygen followed the law of mass action as formulated for homogeneous solutions. These results suggest that the shape of the curve is controlled by the relative amounts of pigment present in the form of salt and in the uncombined state.

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CXVII. THE INFLUENCE OF GLUTATHIONE ON THE OXIDATION OF FATS AND FATTY ACIDS.

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Hopkins [1925] has investigated the influence of glutathione on the oxidation of fats and proteins. The question of the oxidation of the fats and fatty acids is not so straightforward as previously pictured, and so far the problem of the actual mechanism of the reaction has not been reached. It seems, however, desirable to record some of the results which have been obtained.

The work dealt with in the present communication concerns the oxidation of linseed oil and linolenic acid.

The principal results of Hopkins' previous work, in so far as they bear on the results here described, may be briefly summarised.

The oxidation of linseed oil and of linolenic acid or of a mixture of fatty acids was studied at neutrality and at about p_H 3·5. The behaviour of the fat or fatty acid was very different at the two reactions studied.

With linolenic acid or mixed fatty acids at neutrality, the oxygen uptake of the system fatty acid + glutathione (GSH) was about twice that required to oxidise the —SH present. At p_H 3·5, however, where the —SH group is comparatively stable, there was a prolonged uptake to many times the value required by the —SH present.

The behaviour of the oil was again different. At p_H 7·6, after a rapid uptake to approximately the value required to oxidise the —SH present, there was a prolonged rectilinear uptake to a total of many times this value. At p_H 3·5, there was an induction period of several hours, during which the uptake was very small, followed by a period of much greater activity.

The results described in this paper are not in entire agreement with the above: on the other hand, it was found that different samples of oil showed varying behaviour, and similar results to the above could only be obtained with certain conditions of the oil.

Experimental.

The oxygen uptakes were measured in a Barcroft differential manometer, and unless otherwise mentioned 20 mg. oil or fatty acid in the form of an emulsion in water or buffer were shaken with varying amounts of glutathione.

Linseed oil. Three different samples of linseed oil were used in this work, and of these only one showed the behaviour previously described, of giving at p_H 7·5 a prolonged rectilinear uptake after the initial rapid uptake due to the oxidation of the glutathione had ceased.

The "active" oil. This sample of oil showed a prolonged rectilinear uptake, after the glutathione was nearly all oxidised, of about 30 mm.^{3} per hour, and the amount of this uptake, in repeated experiments, was found to be independent of the amount of GSH or of G_2S_2 + muscle residue employed. It depended merely on the quantity of oil used. (It should be noted that this uptake is much smaller than that shown in a typical curve in Hopkins' paper.) In water alone, however, as previously found, no uptake was observed in the absence of GSH. In some experiments which were carried out in Clark and Lubs' phosphate buffer, p_H 7·5, a linear uptake at a slightly greater rate, about 40 mm.^{3} per hour, was observed, even in the absence of any thiol compound.

The behaviour of this oil changed considerably on being kept for some time in a corked bottle in a cupboard. The above results were obtained from September to November 1925, but by April 1926 the oil had become much more active, and now showed an uptake of considerable amount even in water alone. The effect of adding glutathione to the emulsion in water was now simply to increase the total uptake of the system by an amount approximately equal to that of the —SH present. The rate of uptake was not changed by this addition (Fig. 1).

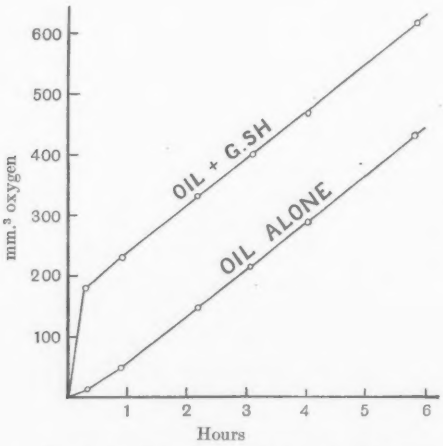

Fig. 1. "Active" linseed oil in water p_H 7·5.

At an intermediate stage (January 1926) during the maturing of this oil, the behaviour was reminiscent of Hopkins' results with the fatty acids. The uptake of a suspension of oil (in buffer) was twice that of the GSH present,

but at the end of this double uptake, a rectilinear uptake continued at the same rate (40 mm³. per hour) as has already been observed. In water, at this time, the initial uptake was considerably greater than that of the GSH present, but it never rose to double this value.

In the later stages (April 1926) after the oil had become active, the effect of GSH in buffer solutions was peculiar. Instead of helping, or at any rate not inhibiting the oxidation of the oil, it seemed that the oxidation was actually slowed down after the GSH had itself become oxidised (Fig. 2). A control experiment with G_2S_2 showed this inhibition from the beginning. This is very like the result found for linolenic acid (see below).

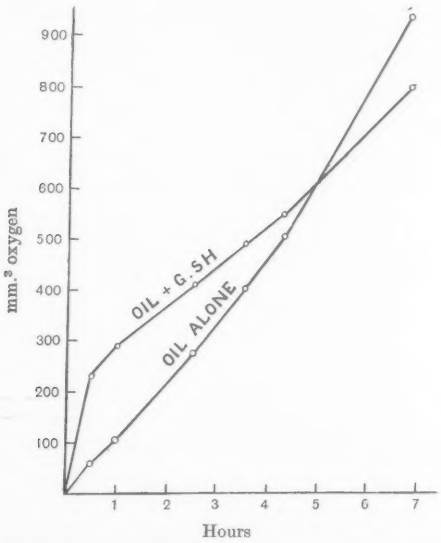

Fig. 2. "Active" linseed oil in phosphate buffer p_H 7·5.

The "inactive" oil. One of the samples of oil which previously had shown no rectilinear uptake was made to do so by aeration for $2\frac{3}{4}$ hours at 100°: by this time the iodine value, originally 180, had fallen to 165. Aeration for 2 hours, which caused no appreciable change in the iodine value, was also ineffective in bringing about an appreciable rectilinear uptake. This is significant in view of Hjort and Lund's work [1925] which is discussed later. At the same time it was noticed that the oil which at the beginning was distinctly yellow in colour, had become much paler: the oil which was "active" when the work commenced was much paler in colour than the "inactive" oil.

Hopkins [1925] says "The longer the induction period displayed by a sample of oil at p_H 3 to 4, the higher the concentration of —SH required to establish an active system at p_H 7·6."

When examined in water suspension at p_H 3·5 no appreciable uptake was observed with this "inactive" oil before aeration, either with or without glutathione. Harrison [1924] has shown the importance of iron in the oxidation of glutathione. Recent samples of glutathione have been much more iron-free than previous specimens, and the experiment was made of adding 0·02 mg. iron (in the form of ferrous ammonium sulphate) to the mixture of fatty emulsion and glutathione. A curve was obtained similar to those previously obtained by Hopkins at p_H 3·5, showing an induction period of $2\frac{3}{4}$ hours. Control experiments, using iron and oil, or iron and glutathione alone at this p_H showed a negligible uptake.

Nevertheless, even with the addition of 0·02 mg. iron, no rectilinear period was obtained with this oil at p_H 7·6 with an amount of glutathione corresponding to 250 mm.^{3} oxygen. The uptake of the system was increased, it is true, by the addition of oil to 364 mm.^{3} but ceased quite definitely after this. (Another inactive sample with the addition of iron at p_H 7·6 showed exactly double the uptake due to —SH present, though without iron the uptake did not reach this value.) After aeration this sample showed the typical curve, with induction period, when examined in water at p_H 3·5 in presence of glutathione.

It is of interest to note that though both the "active" oil and the "inactive" oil after aeration showed behaviour of the same general form as that previously described, the uptake after the induction period was very much smaller than that obtained by Hopkins, unless iron was also added to the system.

Fatty acids. Linolenic acid was prepared on two occasions by the reduction of hexabromostearic acid, obtained by the bromination of the mixture of fatty acids from the saponification of linseed oil, as described by Rollett [1909].

The first sample, immediately after preparation, when tested in water alone at p_H 7·5 showed a very large oxygen uptake. Unfortunately, all attempts to repeat this curve, with the same or different samples of acid were unsuccessful, the acid subsequently showing only a comparatively small uptake in water alone, though an appreciably larger one in buffer.

The results of adding GSH were very similar to those recorded above for the "active" sample of linseed oil. In water at p_H 7·5 the difference in uptake was approximately equal to that of the —SH added: in buffer there appeared, as before, an inhibition after the initial rapid uptake. Again, the addition of G_2S_2 caused inhibition from the start.

It was thought that the increased uptake of the fatty acid or oil in buffer as compared with that in water might be due to the presence of iron in the buffer. The effect of potassium cyanide was therefore tried. In 5 hours 20 mg. of a given sample of linolenic acid took up the following amounts of oxygen.

		mm.^{3}
In phosphate buffer alone		508
" " "	+20 mg. G_2S_2	280
" " "	+6 mg. NaCl	396
" " "	+KCN (to $M/250$)	138

The value found in presence of KCN was only slightly greater than that found in water alone.

At p_H 3·5, however, glutathione caused a definite increase in the uptake of the substance both in water and in phthalate buffer.

A sample of freshly made mixed fatty acids showed a similar behaviour. Nothing approaching the "double uptake" previously described was observed. After the mixed acids had been heated *in vacuo* at 100° from time to time during several days, the spontaneous activity in water was found to have practically disappeared, and now when examined in the presence of glutathione and a trace of iron (0·02 mg.) the characteristic "double uptake" was observed. Without the iron, under identical conditions, the uptake was greater than that of the glutathione by about 50 %. Control experiments showed that this effect was not due to the iron catalysing the oxidation of the mixed fatty acid.

The spontaneous activity of the linolenic acid could not be entirely removed by this means, and although, by the addition of iron to the system, an initial uptake could be obtained much greater than that of the glutathione itself, an accurate "double uptake" was never observed.

Discussion.

The results described above were unexpected, and modify some of the results previously obtained by Hopkins.

The two chief variables appear to be the condition of the oil or fatty acid and the iron content of the system.

Influence of iron. Different preparations of reduced glutathione vary much, not only in their content of GSH, but also in their iron content.

Hopkins [1925, p. 788] says "Since Warburg's discovery, the use of specially pure reagents has yielded preparations containing, at most, infinitesimal quantities of the metal. The employment of these, instead of earlier preparations, has made surprisingly little difference to the experimental results." Preparations since this was written have contained even less iron, and it would seem that the amount they contain is in some cases insufficient to produce a properly active system. The result with the "inactive" linseed oil at p_H 3·5 is perhaps the clearest example of this. Only in the presence of glutathione *and* iron is the typical curve developed; with neither of these separately is this result obtained.

Although Hopkins found that the use of buffers greatly reduced the uptake of a washed muscle preparation, it was not thought likely that the use of buffer would influence materially the oxidation of the oil and fatty acid, and with a view to regulating more exactly the p_H of otherwise quite unbuffered fluids, such as the suspension of the oils and fatty acids, buffers were employed in some of the experiments[1].

[1] It was found in some experiments in water, when the uptake was followed for a long time, say to 1500 or 2000 mm.^{3} per 20 mg., that the reaction of the suspension had definitely changed towards the acid side by the end of the experiment.

They were, however, found to have a marked influence on some of the results.

Both at p_H 3·5 and p_H 7·6 the use of buffer seems to increase the rate of uptake of an active oil or fatty acid: at p_H 7·6 the peculiar apparent inhibition by G_2S_2 must be considered. The results described in the experimental part seem to show that two factors may possibly be involved. The reaction is very markedly slowed by the addition of cyanide, which points to the presence of iron as the cause of the acceleration in buffer. The inhibition by G_2S_2 may possibly be due to the formation of some complex in which the iron is in some less active state. It was found, however, that the reaction was slightly inhibited by the presence of neutral salts, such as sodium chloride, and there is therefore the possibility that the action of glutathione in this connection may be, in part, due to "salt action."

Fats and fatty acids. The fact clearly emerges from the present work that the behaviour of a given sample of oil changes slowly on keeping. It was realised previously [Hopkins, 1925, p. 796] that the behaviour of a given sample of oil can be changed by previous treatment such as heating *in vacuo*, aeration, etc., but unfortunately, no initially "inactive" specimen of linseed oil was encountered during the course of that work.

The work of Hjort and Lund [1925] bears on this point. These authors investigated the oxygen uptake of cod-liver oil at 100°. Starting with perfectly fresh cod-liver oil, there was a long induction period of about 10 hours, during which the oil had a total uptake of only about 0·5 cc. per g. During the next hour or so the uptake rose to about 10 cc. per g. per hour. This long induction period would correspond to something of the order of a year at room temperature, assuming the rate of reaction to be doubled for 10° rise of temperature. It seems possible that the difference between the previous results of Hopkins and those described in this paper may be due to differences in the "maturity" of the oils used. The "active" oil used in the work here described may be assumed to have been near the end of Hjort and Lund's induction period at the beginning of the work, and to have reached the more active condition of those authors by the later part of the investigation. The "inactive" oil may be assumed to be somewhere on the earlier part of Hjort and Lund's curve, and not sufficiently near the end of the induction period to become active without previous aeration. The "active" oil and the "inactive" after aeration showed a marked tendency to harden whilst before aeration the "inactive" oil showed no tendency to harden on exposure to air.

Coffey [1921] has studied the oxidation of linseed oil, but used for his work thin films of the oil on filter papers. A definite induction period was found, which was much shorter than that obtained by Hjort and Lund with cod-liver oil. The work of the latter authors, who used the oil in much greater bulk, is probably more applicable to the changes likely to occur on keeping the oil in a bottle.

It seems quite clear that, given the right conditions, glutathione can induce

at p_H 7·5 a coupled oxidation of either the oil or fatty acid, causing them to take up an amount of oxygen equal to that taken up by the glutathione itself. As far as can be seen, the requisite conditions seem to be (*a*) the oil or fatty acid used must not show great spontaneous activity, (*b*) a definite amount of iron is necessary in the system. There may be other factors which are not at present realised.

How much the glutathione is responsible for the rectilinear uptake at p_H 7·5 is not so clear. The fact that the rate of uptake seems independent of the amount of —SH present, and that uptake at the same rate occurs in buffer in the absence of glutathione, would seem to rule out glutathione as a cause of this. The reason for the absence of uptake in water alone in the earlier experiments is obscure. In the later experiments, when the oil had become really active, an uptake was constantly found in water in the absence of glutathione.

As regards the fatty acids, the spontaneous activity of these is not in accord with Meyerhof's remarks [1923, p. 547].

It is true that glutathione exerts its influence most clearly at the acid reaction, and that with an already active oil or acid the influence of glutathione is not obvious at neutrality; but under certain conditions, the glutathione can undoubtedly induce a coupled oxidation of the oil or fatty acid.

Coffey [1921, p. 1160] makes reference to the common opinion that the fatty acids are less easily oxidised than the oils and states that his experience is otherwise. In neither his work nor in that of Hjort and Lund do the free fatty acids show any definite induction period. The work described above bears out the fact that the acids themselves are more easily oxidised.

Summary.

The influence of glutathione on the oxidation of linseed oil and of linolenic acid has been studied.

The principal results are as follows:

(1) Not all samples of linseed oil or linolenic acid give the results described by Hopkins.

(2) The behaviour of the fatty acids was found to be much more like that of the glycerides than would appear from previous work.

(3) A given sample of oil undergoes changes merely on keeping under ordinary conditions, becoming more "active" as time goes on. The same increase in the "activity" of a sample of oil can be produced by aeration.

(4) Some doubt is thrown on the part played by glutathione in establishing a rectilinear uptake with linseed oil at p_H 7·5.

(5) The "double uptake" previously obtained with the fatty acids at p_H 7·5 seems to depend upon the lack of spontaneous activity of the fatty acid and the iron content of the system, and possibly other factors. Given the right conditions, it can also be obtained with the oils.

(6) Linolenic acid and some samples of linseed oil were found to have a definite uptake in aqueous suspension without the addition of glutathione.

The thanks of the author are due to Professor Sir F. G. Hopkins, at whose suggestion the work was undertaken, for the constant interest he has shown during the course of the investigation.

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CXVIII. THE KINETICS OF PROTEIN DENATURATION.

PART I. THE EFFECT OF VARIATION IN THE HYDROGEN ION CONCENTRATION ON THE VELOCITY OF THE HEAT DENATURATION OF OXYHAEMOGLOBIN.

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The generally accepted view of the heat coagulation of proteins is that of Hardy [1899], who concludes that the process takes place in two stages, the first of which is a preliminary change in the chemical structure of the protein. This stage is termed "denaturation" and is followed by a purely physical process—"flocculation." While Lepeschkin [1922] is of the opinion that flocculation is not entirely physical in nature but to some extent chemical, it is generally agreed that in the presence of electrolytes the second stage takes place at a much more rapid rate than the preliminary stage of denaturation and hence by working in solutions always containing a little electrolyte the kinetics of the heat denaturation of proteins may be studied. That the presence of electrolyte is necessary for the final flocculation is shown by the observation of Aronstein [1874] and Heinsius [1874] that in the absence of electrolytes a protein solution may be heated without flocculation, but that if salt be added after cooling flocculation takes place.

Opinion differs as to the nature of the fundamental chemical process involved in denaturation. Two opposing views are held. The one is that the reaction concerned is a chemical condensation between free amino and carboxyl groups of adjacent molecules. The other view is that the process is a mild hydrolysis. It is obvious, having regard to the relative magnitude of the protein and water molecules, that chemical analysis cannot hope to reveal the slight change in composition occasioned by either of these processes. In fact Hirsch-Pozany [1922] has attempted to differentiate between the two possibilities but without success. It was thought, however, that a study of the kinetics of the reaction and of the effect of various factors on its progress might provide some insight into the nature of the process.

The variation of the velocity of heat denaturation with the hydrogen or hydroxyl ion content of the solution (or more briefly with the p_H of the

solution) is the effect which first suggests itself for study and it is with this, in relation to the heat denaturation of oxyhaemoglobin, that the present paper is concerned.

Chick and Martin [1910] found that while the heat denaturation of oxyhaemoglobin followed the unimolecular law, that of egg-albumin did not, the latter reaction slowing off more rapidly than could be accounted for on such a basis. In later papers [1912] these authors show that when precautions are taken to keep the p_H of the solution constant the velocity of the heat denaturation of egg-albumin conforms to the unimolecular law. They attribute the slowing off in the velocity of heat denaturation to the fact that during heat denaturation of this protein a decrease of hydrogen ion concentration occurs in acid solution and of hydroxyl ion concentration in alkaline solution.

As Chick and Martin found it possible to obtain a steady unimolecular velocity constant for the heat denaturation of oxyhaemoglobin it has been implicitly assumed that no change of p_H takes place during the process. This has been verified during the course of the present work.

This, however, does not preclude variation in the rate of denaturation with p_H. Accordingly, experiments were conducted in which the velocity of the heat denaturation of oxyhaemoglobin was determined in solutions of various hydrogen ion concentrations. The results so obtained are recorded in the following section.

Experimental.

Materials used. The haemoglobin was prepared from fresh defibrinated ox-blood by a modification of the method of Schulz [1898]. The corpuscles were first centrifuged off and washed four times with isotonic salt solution, following which they were laked with distilled water and the haemoglobin obtained by fractional crystallisation by means of ammonium sulphate. The crystallisation was carried out five times and the resulting haemoglobin dissolved and dialysed thoroughly, the solution being constantly brought to the isoelectric point of the protein. In this way, a stock solution very rich in haemoglobin and containing very little salt was obtained. The actual composition of each stock solution was determined, the protein being estimated by the method of Devoto as modified by Hopkins [1900]. This consists in the gravimetric estimation of the coagulum formed on heating a known volume of the solution for two hours in the presence of saturated ammonium sulphate. After coagulation of the protein at the isoelectric point followed by washing and boiling of the coagulum with distilled water, the salt solution so obtained was boiled down to a very small bulk and the salt estimated gravimetrically as sulphate. As no other preservative was admissible a small, known amount of pure ammonium sulphate was added to the stock solution which was kept in the ice-chest.

The ammonium sulphate used was Merck's "puriss." thrice crystallised. The water used was well-boiled CO_2-free water which was tested and found to be free from inorganic and organic impurities and to be of p_H 5·8 at 25°.

The solutions for use were made up by diluting the stock solution of isoelectric protein appropriately with water containing a little NH_4OH or H_2SO_4 as desired. The p_H of the solution was then determined by means of the quinhydrone electrode at 37°[1] following the technique of Corran and Lewis [1924].

After each experiment the p_H was again determined and, as recorded above, was found to be sensibly unaltered.

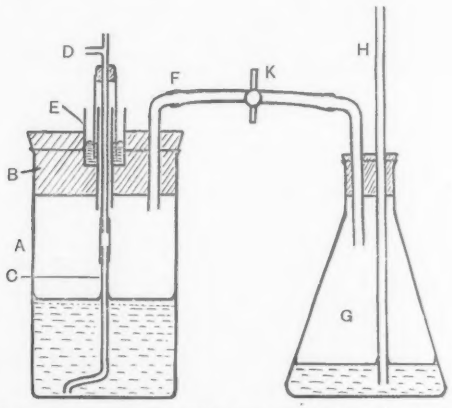

Fig. 1. Apparatus used for the heat coagulation of proteins.

The apparatus employed (Fig. 1) was a modification of that used by Chick and Martin [1910]. The present apparatus is designed to prevent concentration of the solution during the reaction. The reaction vessel, *A*, is a silica beaker of capacity 250 cc., closed by a rubber bung, *B*, bearing a hollow stirrer, *C*, through which samples are withdrawn from time to time by way of a side arm, *D*. The bearing of the stirrer is mercury-sealed, *E*, thus preventing evaporation at this point. Air enters the vessel by a side arm, *F*, which leads from a flask, *G*, containing a solution of the same salt concentration and p_H as that under investigation. Air entering this flask does so by way of a vertical tube, *H*, which dips under the surface of the liquid. As an additional safeguard the screw clip, *K*, is kept closed save at the time of taking a sample. Both beaker and flask are kept well immersed in the water of the thermostat which is regulated to $\pm$ 0·02°. This apparatus is found to prevent any serious change of concentration during an experiment. The speed of the stirrer varied from 60 to 70 R.P.M.

As the samples of solution were withdrawn from the reaction vessel they were rapidly cooled in ice and it was estimated that the whole operation from the stopping of the stirrer motor to the reduction of the solution to ice cold

[1] The temperature 37° was selected as it was desired to work as near to the temperature of the experiments as was feasible. Although the p_H of the solution at the higher temperature would certainly be different from what it was at 37° yet working always with reference to one particular temperature the solutions used at various temperatures were more comparable.

occupied 35 seconds. The stirrer motor was off for a period of 4 to 5 seconds each time a reading was taken. In order to render the time readings more accurate—an important factor in rapid coagulation—each operation was carefully timed to the nearest second.

The samples so obtained were then filtered free from coagulum and the residual haemoglobin estimated by means of a Bausch and Lomb biological colorimeter.

Results.

Section 1. *Heat denaturation of* 1 % HbO_2 *containing* 0·5 % *ammonium sulphate at various* p_H *values at* 60·5°.

Table I.

	p_H	Time in minutes	Residual HbO_2 %	$k_{uni} \times 10^4$	
(i)	5·69	0	100	—	Mean k_{uni} $4·4 \times 10^{-4}$.
		5	87·7	4·4	Mean k_{uni} of duplicate experiment
		10	77·8	4·5	$4·4 \times 10^{-4}$.
		20	60·5	4·4	Average k_{uni} $4·4 \times 10^{-4}$.
		30	47·8	4·3	
		50	29·5	4·1	
(ii)	5·81	0	100	—	Mean k_{uni} $3·32 \times 10^{-4}$.
		10	81·5	3·32	Mean k_{uni} of duplicate experiment
		20	67·1	3·31	$3·32 \times 10^{-4}$.
		30	54·4	3·35	Average k_{uni} $3·32 \times 10^{-4}$.
		50	36·7	3·30	
		80	20·4	3·32	
(iii)	6·05	0	100	—	Mean k_{uni} $2·04 \times 10^{-4}$.
		10	88·0	2·12	Mean k_{uni} of duplicate experiment
		20	78·4	2·02	$2·05 \times 10^{-4}$.
		30	69·0	2·06	Average k_{uni} $2·04 \times 10^{-4}$.
		60	48·0	2·01	
		100	29·7	2·02	
(iv)	6·43	0	100	—	Mean k_{uni} $1·25 \times 10^{-4}$.
		20	86·2	1·24	Mean k_{uni} of duplicate experiment
		50	68·5	1·28	$1·24 \times 10^{-4}$.
		100	47·1	1·25	Average k_{uni} $1·25 \times 10^{-4}$.
		150	32·7	1·24	
		200	22·4	1·24	
(v)	6·83	0	100	—	Mean k_{uni} $1·05 \times 10^{-4}$.
		20	88·1	1·06	Mean k_{uni} of duplicate experiment
		40	78·1	1·04	$1·06 \times 10^{-4}$.
		100	53·7	1·04	Average k_{uni} $1·05 \times 10^{-4}$.
		160	37·2	1·06	
		220	25·8	1·07	
(vi)	7·02	0	100	—	Mean k_{uni} $1·24 \times 10^{-4}$.
		20	86·2	1·24	Mean k_{uni} of duplicate experiment
		50	68·6	1·25	$1·25 \times 10^{-4}$.
		100	47·2	1·25	Average k_{uni} $1·24 \times 10^{-4}$.
		150	32·6	1·24	
		200	22·4	1·24	
(vii)	7·21	0	100	—	Mean k_{uni} $1·67 \times 10^{-4}$.
		20	81·7	1·69	Mean k_{uni} of duplicate experiment
		40	67·5	1·70	$1·67 \times 10^{-4}$.
		80	45·4	1·66	Average k_{uni} $1·67 \times 10^{-4}$.
		120	30·9	1·64	
		180	17·3	1·65	

Table I (*continued*).

	p_H	Time in minutes	Residual HbO_2 %	$k_{uni} \times 10^4$	
(viii)	7·40	0	100	—	Mean k_{uni} $2·20 \times 10^{-4}$.
		10	87·4	2·24	Mean k_{uni} of duplicate experiment
		20	76·9	2·19	$2·20 \times 10^{-4}$.
		30	67·1	2·20	Average k_{uni} $2·20 \times 10^{-4}$.
		50	51·3	2·18	
		80	34·4	2·19	
(ix)	7·60	0	100	—	Mean k_{uni} $2·67 \times 10^{-4}$.
		10	85·1	2·68	Mean k_{uni} of duplicate experiment
		20	72·5	2·69	$2·68 \times 10^{-4}$.
		40	52·4	2·67	Average k_{uni} $2·67 \times 10^{-4}$.
		60	38·2	2·67	
		100	20·2	2·66	
(x)	7·96	0	100	—	Mean k_{uni} $3·33 \times 10^{-4}$.
		10	81·7	3·31	Mean k_{uni} of duplicate experiment
		20	66·6	3·33	$3·33 \times 10^{-4}$.
		40	44·8	3·31	Average k_{uni} $3·33 \times 10^{-4}$.
		60	30·5	3·35	
		80	20·6	3·33	
(xi)	8·32	0	100	—	Mean k_{uni} $3·7 \times 10^{-4}$.
		10	79·4	3·7	Mean k_{uni} of duplicate experiment
		20	60·6	3·7	$3·7 \times 10^{-4}$.
		40	40·8	3·8	Average k_{uni} $3·7 \times 10^{-4}$.
		60	26·4	3·7	
		80	17·0	3·7	

Table II. *Summarising table. Heat denaturation of* 1 % HbO_2 *containing* 0·5 % *ammonium sulphate.*

Variation of k_{uni} with p_H at 60·5°.

p_H	$k_{uni} \times 10^4$
5·69	4·40
5·81	3·32
6·05	2·04
6·43	1·25
6·83	1·05
7·02	1·24
7·21	1·67
7·40	2·20
7·60	2·68
7·96	3·33
8·32	3·70

The data of Table II are plotted in Fig. 2. These results bring out an interesting behaviour on the part of the protein. They show that the velocity of denaturation passes through a minimum as p_H changes and it is not, as might have been expected, protein which was originally isoelectric which shows this minimum velocity but that which at 37° has a p_H of 6·76, the p_H of isoelectric oxyhaemoglobin at this temperature being 6·5 [Warburg, 1922; Stadie and K. A. Martin, 1924][1].

[1] Although the p_H of the solution as measured by the quinhydrone electrode is strictly a measure of the activity of hydrogen ion, it may be taken that in the solutions employed here activity of hydrogen ion is sensibly equal to concentration of hydrogen ion owing to the solutions being dilute with respect to hydrogen ion.

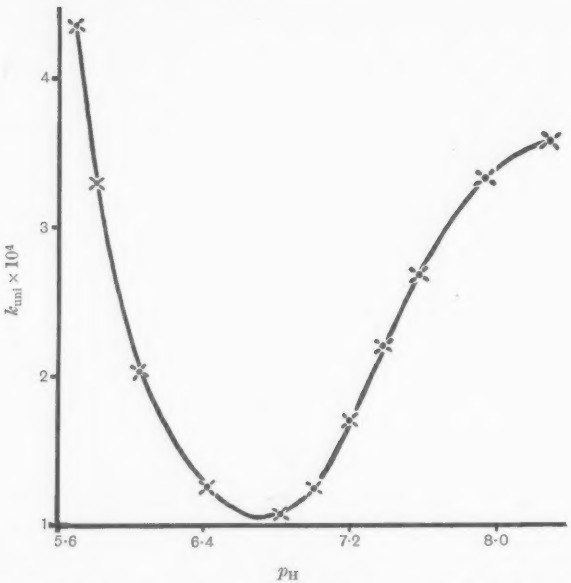

Fig. 2. Heat denaturation of oxyhaemoglobin. Variation with p_H of k_{uni} at 60·5°.

SECTION 2. *Heat denaturation of* 1 % HbO_2 *containing* 0·5 % *ammonium sulphate at various* p_H *values at* 68°.

The results shown below are, in each case, the mean values of at least two experiments. The order of accuracy is similar to that of the previous section. Fuller details are omitted for the sake of brevity.

Table III. *Summarising table.*

Variation of k_{uni} with p_H at 68°.

p_H	$k_{uni} \times 10^3$
5·74	5·1
5·99	2·96
6·32	1·83
6·55	1·43
6·75	1·32
6·95	1·49
7·02	1·59
7·16	2·04
7·46	3·04
8·05	4·45

The data of Table III if plotted yield a graph similar to Fig. 2. Here again the velocity of denaturation passes through a minimum at p_H 6·76 and rises more rapidly on the acid than on the alkaline side. As the p_H was determined at 37° in each case this means that the same solution which denatures with the minimum velocity at 60·5° will also do so at 68°.

The effect of temperature on the rate of the heat denaturation of oxyhaemoglobin.

The first workers in this field were Chick and Martin [1910] who found for this reaction a critical increment[1] of 60,000 calories. Later, Hartridge [1912] found values of 74,000 calories and 86,000 calories which confirms the order of the result of Chick and Martin.

The most noteworthy point about this critical increment is its high numerical value, from which it appears to be a composite term, the sum of several smaller critical increments, for a true critical increment of this magnitude would only be expected to accompany a process which would be immeasurably slow. This conclusion is based on general experience of the magnitude of critical increments characteristic of processes which occur with measurable velocities.

Chick and Martin say that the haemoglobin used by them was "used as prepared and contained ammonium sulphate." Further, they do not specify at what p_H they worked. The same applies to the results of Hartridge. The writer has found that both of these variables have a great effect on the velocity of denaturation. It is essential therefore, to make the comparison at the same p_H.

The critical increment has been calculated in the present instance from the minimum velocities on the p_H/k_{unt} curves. The value so obtained is 77,500 calories. It will be shown in Part III that this value is in good agreement with those obtained by two other methods.

Discussion of results.

The term denaturation is used in the literature to describe a large variety of processes whereby a protein may be converted from a soluble to an insoluble form. Such processes include the action of alkalis, acids, heavy metal salts, alkaloidal reagents, heat, light, pressure, friction, etc. There does not seem much ground for supposing that all these various processes are fundamentally the same.

Chick and Martin [1910] used the term to designate the chemical reaction which precedes flocculation on heating a solution of heat-coagulable protein. They consider this chemical reaction as taking place between the protein and hot water, and are of the opinion that it takes the form of a slight hydrolysis. This same view is taken by other authors, as Lepeschkin [1918], Wu and Wu [1925], etc.

[1] This is calculated from the integrated form of the Marcelin-Rice equation:

$$\log_e k_1 - \log_e k_2 = \frac{E}{R} \cdot \frac{T_1 - T_2}{T_1 \cdot T_2}$$

where k_1 and k_2 are the velocity constants of the reaction at the absolute temperatures T_1 and T_2, E is the critical increment or energy of activation of the process and R is the gas constant. Actually the above authors determined the μ term of Arrhenius. The expression used for the calculation of μ is $\log_e \frac{k_1}{k_2} = \frac{\mu}{2}\left(\frac{T_1 - T_2}{T_1 \cdot T_2}\right)$ which is identical in form with that used for the calculation of E; μ taking the place of E and the empirical figure 2 that of the gas constant R. Thus the μ value of a process will be about 1 % higher than the corresponding critical increment.

Robertson [1918], on the other hand, considers the reaction to be one of dehydration, a view originally put forward by Hofmeister [1888].

There exists also the possibility of intramolecular rearrangement. Excluding this, for which there is no experimental evidence, we are left to decide between hydrolysis (or possibly hydration) and dehydration (chemical or physical) as the underlying chemical reaction in heat denaturation.

Robertson [1918] argues from the following evidence. He himself found [1911] that on the addition of a fairly concentrated solution of cupric chloride to a solution of caseinogen in anhydrous formic acid the mixture is at first green, indicating the presence of lower hydrates of cupric chloride, but on adding more of the solution it becomes blue and simultaneously with the appearance of a pure blue colour, but not before, precipitation of cupric caseinogenate occurs. Further, if to the green solution water be added until the pure blue colour of fully hydrated cupric chloride is shown, the precipitate again forms. If formic acid be added to the mixture the precipitate dissolves as soon as the mixture becomes green. Further, the precipitate dissolves on warming, the mixture becoming green; on cooling the blue colour returns and with it the precipitate.

If instead of adding water to the green solution alcohol be added, no precipitation occurs until the colour of the mixture changes to the brown of the anhydrous salt.

Since water and alcohol are respectively hydrating and dehydrating agents, he concludes that proteins may be thrown out of solution in two grades of hydration, the one of high and the other of very low hydration. The former process he terms precipitation and the latter coagulation.

He then says that "dehydration partial or complete, leading to resolution or coagulation, may be induced by heat, by non-electrolytes possessing an affinity for water or by electrolytes."

It is doubtful, however, whether the evidence just cited can be justly applied to the phenomenon of heat denaturation. In Robertson's experiments the precipitate was one of cupric caseinogenate and the precipitation was reversible, whereas in heat coagulation the protein is precipitated as such and the process is, so far as one can determine, irreversible.

Robertson [1918], however, argues that the irreversibility of heat coagulation is only apparent and cites the observation of Corin and Ansiaux [1891] that if a solution of protein be cooled and vigorously shaken, just as the first traces of heat coagulation appear, the incipient coagula will again pass into solution. The writer has repeated this experiment with both egg-albumin and haemoglobin without success. A similar failure is recorded by Wu and Wu [1925] who, in addition, heated a solution containing the minimal quantity of acid or alkali required to prevent coagulation and cooled and kept the solution for two months in an ice-box under sterile conditions. The solution was then neutralised and the protein precipitated. It appears, therefore, that the protein cannot revert to its original condition and the process is accordingly irreversible.

Robertson considers that there exists between the protein and water an equilibrium of the type

$$H.X.OH + H.X.OH \rightleftarrows HX.XOH + H.OH$$

and that the effect of increasing the temperature of the system is to displace the equilibrium from left to right. This he concludes from the fact that, at temperatures higher than 40°, there is an increase in the solubility in alkaline solutions [Robertson, 1908] and in the alkalinity and electrical conductivity [Osborne, 1901] of caseinogen.

Robertson's explanation of these facts is quite feasible and may be correct yet it seems to the writer that this increased solubility is fictitious, and in fact breakdown of the protein rather than polymerisation suggests itself. More particularly so as Robertson did not observe any tendency to reprecipitation on cooling on the part of the excess caseinogen dissolved at the higher temperature. Granting, however, the correctness of Robertson's interpretation, the transfer of these conclusions to the process of heat denaturation does not seem justifiable. In fact caseinogen, which, save in alkaline solution, is relatively insoluble [Osborne, 1901] and the solubility of which increases on heating, hardly seems a good subject for the investigation of the phenomenon of heat denaturation. It must be emphasised that, when dealing with a molecule so complex as that of the protein, changes may be observed on heating the aqueous solution other than that particular change which we term denaturation. Thus Sørensen [1925] shows that on heating a solution of egg-albumin, hydrolytic scissions take place which, as they do not proceed hand in hand with coagulation cannot be the fundamentally important processes in denaturation. It therefore seems necessary to restrict observations to the actual process of heat denaturation.

That water is necessary for the heat denaturation of proteins might be inferred from the observation of Wichmann [1899] that dry albumin may be heated to 150° for some time without change. This is confirmed by Chick and Martin [1912] who heated dry egg-albumin to 120° for five hours without change although in the presence of steam it was denatured. Robertson argues, however, that this is because in the absence of water the terminal NH_2 and COOH groups of the protein are not ionised and hence no action can take place.

The noteworthy point about the results recorded in the present paper is that they show that protein which, at 37°, has a p_H of 6·76 is denatured with the minimum velocity. Now 6·76 is the p_H of "neutral" water at 37°, that is, in this solution the concentrations of hydrogen and hydroxyl ions are equal. On heating the solution to the temperature of the experiment the p_H will of course change, but it is thought that this equality of ionic concentrations will still hold. That the relative concentrations of these two catalysing ions does not change appreciably at the higher temperature is shown by the fact that the same solution has the minimum velocity of denaturation at both 60° and 68°. It is not feasible to determine the p_H of the solution at the high temperature, but in order to make more certain of this point the p_H of a haemo-

globin solution, which was of p_H 6·76 at 37°, was determined at 25°, 18° and 0° with the following results.

Table IV. *Variation with temperature of the p_H of a solution containing 1 g. of haemoglobin and 0·5 g. $(NH_4)_2SO_4$ per 100 cc.*

Temp. in degrees C.	p_H of solution	p_H at which $H^+ = OH^-$	Authority
37	6·76	6·76	Lorenz and Bohi [1909]
25	6·99	6·99	Lewis, Brighton and Sebastian [1917]
18	7·09	7·08	Kohlrausch and Heydweiler [1894]
0	7·51	7·45	

From these results it appears that in the solution containing protein and ammonium sulphate, the concentration of hydrogen ion is sensibly the same as that of hydroxyl ion over a range of 37 degrees and it is thought that it is quite likely to be the case up to 60° or 70°.

The reaction postulated by Robertson as the basis of denaturation, *i.e.* that of the chemical condensation of free amino and carboxyl groups, would have a maximum probability at the isoelectric point of the protein, as here the two groups he considers are dissociated to an equal extent. As the solution departs more and more from the isoelectric condition the dissociation of one or other of these groups becomes very rapidly depressed and on Robertson's view the reaction should be extremely slow in strongly acid or alkaline solution. This is entirely different from the behaviour observed.

That the variation of the velocity of denaturation with p_H is not connected with the ionisation of the protein *per se* is shown by the fact that the minimum velocity does not occur at the isoelectric point of the protein but at a p_H at which the concentrations of hydrogen and hydroxyl ions are equal. It might be suggested, in view of the slight difference between these two p_H values in the case of haemoglobin, that actually the solution which at 37° was of p_H 6·76 might be isoelectric at higher temperatures. This consideration is ruled out by the fact that the velocity of denaturation of egg-albumin passes through a minimum at the same p_H (viz. 6·76) (see Part II), the isoelectric point of egg-albumin being p_H 4·84 [Sørensen, 1917, 2].

In order to explain the rapid rise of velocity of denaturation with increasing hydrogen and hydroxyl ion concentration on either side of the minimum it is necessary to assume that the reaction is catalysed by these ions. This suggests that the process under consideration is one of hydrolysis, hydrogen and hydroxyl ion catalysis being very typical of this type of reaction.

If these were the sole influences operating, the p_H/k_{unl} curve should be symmetrical about the minimum point[1] which is contrary to experimental observation.

[1] It would follow in this case, since the initial concentration of protein is kept constant, that we could express the velocity thus:

$$\text{velocity} = k_1 C_{H^+} + k_2 C_{OH^-} \qquad \ldots\ldots(1),$$

and hence

$$\frac{d \text{ velocity}}{d C_{H^+}} = k_1 + k_2 \frac{d C_{OH^-}}{d C_{H^+}};$$

In general shape the curve bears a strong resemblance to those obtained by Loeb [1922] for the variation with p_H of viscosity, swelling, and osmotic pressure of proteins in solution, save that the minima in the latter cases occur at the isoelectric point of the protein, which, however, is not far removed from the minimum in the *present* case. It will be seen (Part II) that, in the case of the heat denaturation of egg-albumin, where the isoelectric point of the protein is far removed from the minimum point, the asymmetry is not so pronounced.

To account for the asymmetrical nature of the curve it is suggested that the reaction is in fact a hydrolysis catalysed by hydrogen and hydroxyl ions, water being likewise involved, *the water which is effective being the water of hydration of the protein micelle*[1]. This will vary with p_H in the same manner

at the minimum point this differential is zero, *i.e.*

$$k_1 = -k_2 \frac{d\,C_{OH^-}}{d\,C_{H^+}};$$

but since $C_{H^+} \times C_{OH^-}$ is a constant,

$$\frac{d\,C_{OH^-}}{d\,C_{H^+}} = -\frac{C_{OH^-}}{C_{H^+}}.$$

Hence at the minimum

$$\frac{C_{OH^-}}{C_{H^+}} = \frac{k_1}{k_2}.$$

But the minimum occurs at a point where $C_{H^+} = C_{OH^-}$.

Therefore

$$k_1 = k_2 = k.$$

Equation (1) may now be written

$$\text{velocity} = k\left(C_{H^+} + \frac{K_W}{C_{H^+}}\right) \qquad \ldots\ldots(1\,\text{A}).$$

If the minimum velocity (*i.e.* the point at which hydrogen ion concentration equals hydroxyl ion concentration) occurs at $p_H = N$, then the ionic product of water (K_W) $= 10^{-2N}$. At any other p_H value, say that represented by $p_H = (N + x)$,

$$\text{velocity} = k\left[10^{-(N+x)} + \frac{10^{-2N}}{10^{-(N+x)}}\right] = k\,[10^{-(N+x)} + 10^{-(N-x)}],$$

and at $p_H = (N - x)$

$$\text{velocity} = k\left[10^{-(N-x)} + \frac{10^{-2N}}{10^{-(N-x)}}\right] = k\,[10^{-(N-x)} + 10^{-(N+x)}].$$

Thus the velocity at any two p_H values equidistant from the minimum will be equal. That is, the curve would be symmetrical about the minimum point.

[1] Precisely how the water term will enter into the analytical expression for the speed of the reaction is somewhat uncertain.

If it be taken that the concentration of the water of hydration enters the expression for the velocity of denaturation in the following manner

$$\text{velocity} = (k_1 C_{H^+} + k_2 C_{OH^-})\, C_{H_2O},$$

then by treatment similar to that given above, the expression yields the relationship

$$\frac{k_1}{k_2} = \frac{\left(C_{H_2O} - C_{H^+}\dfrac{d\,C_{H_2O}}{d\,C_{H^+}}\right)}{\left(C_{H_2O} + C_{H^+}\dfrac{d\,C_{H_2O}}{d\,C_{H^+}}\right)}$$

at the minimum point of the p_H/k_{uni} curve.

As the minimum point under consideration is on the alkaline side of the isoelectric point $\dfrac{d\,C_{H_2O}}{d\,C_{H^+}}$ is negative. Hence k_1 is now greater than k_2.

The inclusion of the water term thus renders the p_H/k_{uni} curve asymmetrical about the minimum point and further since $k_1 > k_2$ the curve will fall more steeply on the acid side of the minimum than it rises on the alkaline side; the shape of the hypothetical curve is therefore the same as that observed.

as the viscosity, etc., of the solution, but will be in thermodynamic equilibrium with the total external water of the solution. The suggestion that the water of hydration of the protein is involved appears more likely in view of the results of Part III which are concerned with the variation of k_{uni} with the concentration of neutral salt in the solution.

The balance of the evidence so far available would appear to be in favour of a hydrolysis as the fundamental reaction in denaturation.

On *a priori* grounds it might be thought that the polypeptide link (—CO.NH—) would be the important one for such a reaction. However, work by A. I. Escolme in this laboratory (as yet unpublished) shows that the —CO.NH— link is extremely stable to hydrolysis and in view of the ease with which protein denaturation occurs the latter effect cannot be ascribed to the hydrolysis of this linkage. Further, mere dehydration of a molecule or micelle would be a process corresponding to a relatively low critical increment. The actual observed speed of denaturation would seem to involve a process of intermediate critical increment value.

Lepeschkin [1922] suggested that the chemical reaction involved in the denaturation of proteins by heat is of the same nature as the hydrolysis of polysaccharides into their constituent units. If this be so, it is necessary to assume that several such cleavages are occurring in the denaturation of the protein. The observed critical increment of the hydrolysis of sugars having the sucrose type of linkage is approximately 26,000 calories. So that here we may be dealing with a quantity of the order of 20,000 to 30,000 calories. If the suggestion of Lepeschkin is correct then, from the magnitude (77,500 cals.) of the critical increment observed for the heat denaturation of oxyhaemoglobin, it would be inferred that the reaction consists in the rupture of three or four linkages analogous to that involved in the hydrolysis of sucrose.

To avoid the difficulty which statistical considerations would introduce if denaturation were regarded as the result of collisions of three or four individuals it would seem necessary to assume that large, heavily hydrated units are already in existence in undenatured protein and that in the act of denaturation each unit underwent alteration at three or four places.

In this connection it is very suggestive that Adair [1925] and Svedberg and Fahraeus [1926] have shown, the one by osmotic pressure measurements and the others by means of the ultracentrifuge, that the molecular weight of haemoglobin in solution is four times the minimum weight as determined by analytical means.

Summary.

The influence of variation in the hydrogen ion concentration on the velocity of the heat denaturation of oxyhaemoglobin has been studied. It is found that the velocity passes through a minimum, namely at that point at which the hydrogen ion and the hydroxyl ion concentrations of the solution are equal.

The critical increment of the process is found to be 77,500 calories. It is

pointed out that this value is in all probability a composite term, the sum of several smaller critical increments.

The possible mechanism of the process is considered in the light of current theories. It is suggested that the mechanism consists of a localised hydrolysis of relatively labile links (nature unspecified) situated at various points on a large, heavily hydrated molecular unit. It is considered that the cause of the variation of k_{uni} with p_{H} is the catalytic influence of hydrogen and hydroxyl ions.

In conclusion the writer desires to express his thanks to the Department of Scientific and Industrial Research for a maintenance grant which enabled this investigation to be carried out.

REFERENCES.

The references will be found at the end of Part III, p. 992.

CXIX. THE KINETICS OF PROTEIN DENATURATION.

PART II. THE EFFECT OF VARIATION IN THE HYDROGEN ION CONCENTRATION ON THE VELOCITY OF THE HEAT DENATURATION OF EGG-ALBUMIN; THE CRITICAL INCREMENT OF THE PROCESS.

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(*Received August 4th, 1926.*)

The first work carried out on the rate of the heat denaturation of egg-albumin was that of Chick and Martin [1910], who found that the reaction velocity slowed off more rapidly than was accounted for by the unimolecular law. They also found that the hydrogen ion concentration (in acid solution) and the hydroxyl ion concentration (in alkaline solution) were reduced during the course of the reaction[1], and to this they attributed the falling off of reaction velocity mentioned above. This alteration of the hydrogen ion concentration (or more briefly the p_H of the solution) has been confirmed by Quagliariello [1912], Sørensen and Jurgensen [1911], and more recently by Wu and Wu [1925]. In later papers, Chick and Martin [1912] show that when precautions are taken to keep the p_H of the solutions constant (by saturation with boric acid in acid solution and with magnesium oxide in alkaline solution) a steady unimolecular velocity constant is obtained for the process.

In the present work the solutions were first maintained at a constant p_H by means of buffers but it was found that while a steady unimolecular constant could be obtained in this manner, the actual velocity of denaturation was much enhanced, which is in agreement with the observation of Zoller [1921] that phthalate buffers lower the coagulation temperature of egg-albumin. Further, in view of the finding of Chick and Martin [1910] that the presence of neutral salt lowers the critical increment[2] of the heat denaturation of egg-albumin, the use of buffers was abandoned.

[1] Sørensen [1925] shows that this change of p_H as the reaction proceeds is due to hydrolytic scissions of the protein molecule, which, however, as they do not proceed hand in hand with coagulation, cannot be the fundamentally important processes in denaturation.

[2] This quantity has already been defined in Part I.

Experimental.

The present work has been carried out at temperatures of 65° and 70·2°, in unbuffered solutions but at varying initial p_H values in such a manner that a p_H range of approximately 5 to 8 has been covered.

The method employed was to conduct an experiment in the manner described in Part I and not only to measure the albumin content of each sample withdrawn but also the p_H thereof[1].

The logarithm of the concentration of albumin was then plotted against time. By taking tangents on the resulting curve the unimolecular constants of the reaction at the times corresponding to the determined p_H values were obtained.

By varying the initial p_H of the solution the above-mentioned range has been studied. The logarithms at a given temperature were then plotted against p_H. In this manner a continuous curve is obtained.

The egg-albumin used was prepared by the method of Hopkins and Pinkus as modified by Sørensen [1917, 1]. The estimation of the albumin content of each sample was made by the method of Devoto as given by Hopkins [1900]. This consists in the gravimetric estimation of the coagulum formed on heating a known volume of the solution for 2 hours in the presence of saturated ammonium sulphate.

In all the following experiments the solutions contained initially 1 g. of albumin and 0·5 g. of ammonium sulphate per 100 cc. The velocity constants are expressed in each case in $\log_e$ and sec^{-1}.

Results.

Section 1. *Heat denaturation of egg-albumin at* 70·2°.

Table I.

Time in minutes	Residual albumin in mg. per cc.	$k_{uni} \times 10^3$	p_H
(i) p_H range 4·98 to 5·50 (during the course of the experiment)			
0	5·60	5·1	4·98
1	4·11	3·84	5·16
2	3·35	3·23	5·24
3	2·82	2·46	5·34
4	2·48	2·02	5·43
(ii) p_H range 5·34 to 5·84 (during the course of the experiment)			
0	7·59	2·56	5·34
2	6·03	2·22	5·43
5	4·43	1·62	5·54
7	3·71	1·48	5·58
10	3·24	1·15	5·66
21	1·67	0·45	5·84

[1] p_H measurements were made at 37° by means of the quinhydrone electrode following the technique of Corran and Lewis [1924].

Table I (*continued*).

Time in minutes	Residual albumin in mg. per cc.	$k_{uni} \times 10^4$	p_H
(iii) p_H range 6·20 to 6·48 (during the course of the experiment)			
0	7·27	2·07	6·20
10	6·47	1·79	6·26
30	5·22	1·60	6·32
50	4·42	1·48	6·35
80	3·34	1·33	6·42
125	2·47	0·96	6·48
(iv) p_H range 6·10 to 6·34 (during the course of the experiment)			
0	7·64	3·07	6·10
10	6·57	2·69	6·14
30	4·77	2·27	6·20
60	3·24	2·00	6·24
92	2·25	1·75	6·28
120	1·74	1·29	6·34
(v) p_H range 7·34 to 6·89 (during the course of the experiment)			
0	6·86	2·07	7·34
10	6·05	1·92	7·29
30	4·86	1·60	7·21
50	3·98	1·24	7·12
80	3·43	0·85	7·02
120	2·96	0·55	6·89
(vi) p_H range 7·72 to 7·23 (during the course of the experiment)			
0	6·46	3·84	7·72
10	5·18	3·20	7·65
20	4·42	2·74	7·56
40	3·28	2·13	7·38
60	2·58	1·75	7·26
80	2·09	1·60	7·23

Table II. *Summarising table.*

Variation of k_{uni} with p_H at 70·2°		Variation of k_{uni} with p_H at 70·2°	
p_H	$k_{uni} \times 10^5$	p_H	$k_{uni} \times 10^5$
4·98	510·0	6·34	12·9
5·16	384·0	6·35	14·8
5·24	323·0	6·42	13·3
5·34	250·0	6·48	9·6
5·43	212·0	6·89	5·5
5·50	134·0	7·02	8·5
5·54	162·0	7·12	12·4
5·58	148·0	7·21	16·0
5·66	115·0	7·23	16·0
5·84	45·0	7·26	17·5
6·10	30·7	7·29	19·2
6·14	26·9	7·34	20·7
6·20	21·7	7·38	21·3
6·24	20·0	7·56	27·4
6·26	17·9	7·65	32·0
6·28	17·5	7·72	38·4
6·32	16·0		

The data of this table are plotted in Fig. 1.

It will be seen from these results that the velocity of the heat denaturation of egg-albumin passes through a minimum in the neighbourhood of the point at which the concentration of hydrogen ion is equal to that of hydroxyl ion, namely, at p_H 6·76 measured at 37°.

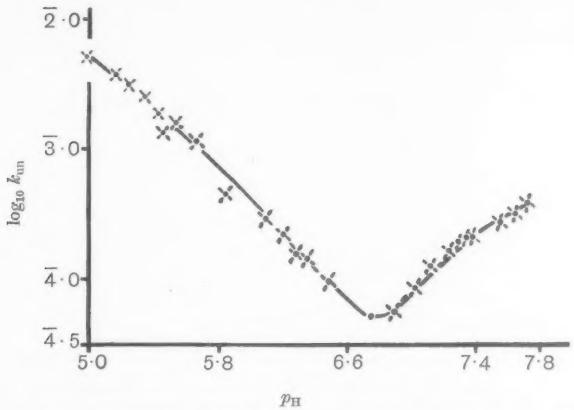

Fig. 1. Heat denaturation of egg-albumin. Variation of k_{uni} with p_H at 70·2°.

It will also be noticed in this case that the asymmetry of the curve is not so pronounced as in the case of oxyhaemoglobin (see Part I). This is attributed to the fact that the minimum is now further removed from the isoelectric point of the protein and hence the hydration of the molecule is varying with p_H to a smaller extent (compare the discussion of results given in Part I).

SECTION 2. *Variation with p_H of the velocity of heat denaturation of egg-albumin at 65°.*

The following table summarises the results of several experiments analogous to those described in the previous section. Fuller details are omitted for the sake of brevity.

Table III. *Summarising table.*

p_H	$k_{uni} \times 10^6$	p_H	$k_{uni} \times 10^6$
4·93	311·0	7·21	5·9
5·02	254·0	7·36	9·7
5·08	205·0	7·42	11·4
5·17	184·0	7·58	16·8
5·30	125·0	7·68	17·3
5·48	56·0	7·73	18·8
5·78	26·7	7·79	20·0
5·89	19·5	7·81	21·3
6·03	14·9	7·83	23·7
6·07	13·7	7·89	24·6
6·13	12·1	7·94	26·7
6·23	9·2	7·99	32·0

The data of this table, when plotted, yield a graph similar to Fig. 1.

Here again the velocity of denaturation passes through a minimum in the neighbourhood of "neutrality." It might therefore be reasonably expected that, if p_H variation were the only cause of the falling off of the velocity, a solution initially at p_H 6·76 (measured at 37°) would remain sensibly steady

in p_H and also give a unimolecular velocity constant. The necessary measurements have been made; the results are recorded in the following section.

SECTION 3. *Heat denaturation of egg-albumin at* p_H 6·76.

Table IV.

Temp.	Time in minutes	Residual albumin in mg. per cc.	$k_{uni} \times 10^5$	
70·2°	0	6·75	—	Mean k_{uni} $5·2 \times 10^{-5}$
	30	6·13	5·4	Mean k_{uni} of duplicate experiment $5·1 \times 10^{-5}$
	80	5·25	5·2	
	120	4·68	5·1	Average k_{uni} $5·15 \times 10^{-5}$
	200	3·72	5·0	This value has been inserted in Fig. 1
	300	2·76	5·0	

Table V.

Temp.	Time in hours	Residual albumin in mg. per cc.	$k_{uni} \times 10^6$	
65°	0	7·77	—	Mean k_{uni} $2·86 \times 10^{-6}$
	5	7·37	2·93	Mean k_{uni} of duplicate experiment $2·74 \times 10^{-6}$
	10	7·00	2·90	
	20	6·32	2·87	Average k_{uni} $2·80 \times 10^{-6}$
	30	5·72	2·84	
	60	4·22	2·82	

The average value of k_{uni} of Table V lies on the curve given by plotting the data of Table III.

The velocity constants obtained above are quite good. The slight fall which is noticed is in all probability due to the p_H employed not being the exact position of the minimum. It seems to be a fair inference from these experiments that denaturation *per se* does not necessarily involve an alteration in p_H as the reaction proceeds.

The important point brought out by these results is that the velocity of the heat denaturation of egg albumin passes through a minimum under conditions where the concentration of hydrogen ion is sensibly equal to that of hydroxyl ion[1]. This is the more interesting in view of the results of Part I of this series, in which it was shown that the heat denaturation of oxyhaemoglobin showed the same behaviour. In the present instance there can be no question of the minimum occurring at the isoelectric point of the protein which is at p_H 4·84 at room temperature [Sørensen, 1917]. In the case of haemoglobin the isoelectric point (p_H 6·5 at 37°) is so slightly removed from neutrality (p_H 6·76 at 37°) that it might have been suggested that the solution which had p_H 6·76 at 37° might be isoelectric at the higher temperature. In view of the present results, however, it may be taken that the conclusions arrived at in Part I are substantially correct.

[1] As the curve (Fig. 1) does not cover the region of the isoelectric point the possibility of there being a *maximum* velocity in the neighbourhood of that point suggests itself. However, experiments at other temperatures (not quoted) show that the velocity of heat denaturation passes continuously through this region without inflexion or change of direction.

The observed critical increment of the heat denaturation of egg-albumin.

For the purpose of calculating the critical increment (cf. Part I) the values used are the velocity constants corresponding to the minimum points on the p_H/k_{unl} curves. From these it is calculated that the apparent critical increment of the process is 130,000 calories. For the same process Chick and Martin [1910] obtained values varying from 128,000 to 141,000 calories. These authors used as a measure of the rate of the reaction the time taken for the concentration of albumin to fall between two definite limits. This method is not so accurate as that employed in the present work and in view of this fact the agreement between the values of the critical increment obtained in the two investigations is excellent.

In Part I of this series it was suggested that the mechanism of the heat denaturation of proteins consisted of a localised hydrolysis of certain links (nature unspecified) situated at various points on a large, heavily hydrated molecular unit. It was shown that the observed critical increment of the heat denaturation of oxyhaemoglobin (78,000 calories) corresponded to the hydrolysis of three or four linkages of a type corresponding to a critical increment of 20,000 to 30,000 calories. It will be seen that the apparent critical increment of the heat denaturation of egg-albumin corresponds to five or six such hydrolysable linkages.

Further, the work of Lüers and Landauer [1922] must be considered. These authors find that for the heat denaturation of leucosin the observed critical increment is 82,500 calories. This also fits in quite well with the magnitudes considered here.

SUMMARY.

The effect of the variation of the hydrogen ion concentration on the velocity of the heat denaturation of egg-albumin has been studied in unbuffered solutions at 65° and 70·2°. It is found that the rate of reaction passes through a minimum at a p_H 6·76 where the concentration of hydrogen ion is sensibly equal to that of hydroxyl ion. It is pointed out that in this instance the asymmetry of the curve is not so pronounced as in the case of the heat denaturation of oxyhaemoglobin and that this lends support to the idea that the water of hydration of the micelle is in some way involved in the reaction.

The critical increment of the heat denaturation of egg-albumin has been found to be 130,000 calories.

It is also shown that the results of Lüers and Landauer for the heat denaturation of leucosin are in general agreement with the results recorded in this paper.

In conclusion the writer desires to express his thanks to the Department of Scientific and Industrial Research for a maintenance grant which enabled this investigation to be carried out.

REFERENCES.

The references will be found at the end of Part III, p. 992.

CXX. THE KINETICS OF PROTEIN DENATURATION.

PART III. THE INFLUENCE OF NEUTRAL SALTS ON THE VELOCITY OF THE HEAT DENATURATION OF OXYHAEMOGLOBIN.

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Aronstein [1874] and Heinsius [1874] first pointed out that if a protein solution be dialysed until free from electrolytes then no coagulation occurs on boiling. If, however, a solution so treated is cooled and a little salt added in the cold, flocculation immediately takes place. From this it is inferred that although the process of denaturation can take place in the absence of salt, yet the salt, presumably as electrolyte, is necessary for the final stage in coagulation, viz. flocculation.

Other early investigations on the effect of neutral salt on proteins are those of Virchow [1854] and of Hofmeister and his pupils [1888, etc.]. These however were mainly concerned with flocculation by salts in the cold, and from the results obtained these authors concluded that inorganic salts threw proteins out of solution by dehydrating them.

Later, Ostwald [1907] showed that certain alkali salts lowered the "coagulation temperature" of egg-albumin. From the temperatures at which he worked, namely 20–40°, it is probable that he was dealing here with flocculation as distinct from denaturation.

Pauli and Handowski [1910] showed that the addition of neutral salts to alkali protein increased the coagulability by heat, the salts of the alkaline earths being more effective than those of the alkali metals. This effect they explained as being due to the depression of the ionisation of the protein owing to the formation of salts of the type $Cl.KH_2N.R.COONa$, thus bringing the properties of the solute nearer to those of the original protein.

The first to distinguish between the action of neutral salts upon flocculation in the cold and upon heat denaturation were Michaelis and Rona [1910] who state that while neutral salts diminish the rate of flocculation in the cold they do not delay the rate of heat coagulation.

Variation of the ammonium sulphate concentration of the solution was then studied by Micko [1911] who found that while the "coagulation temperature of *egg*-albumin was unaffected by the addition of ammonium sulphate that of serum-albumin and of milk-albumin was raised by the addition of ammonium sulphate."

In contrast to this, Chick and Martin [1912] state that both ammonium sulphate and sodium chloride greatly reduce the rate of denaturation of *egg*-albumin and also the critical increment of the process.

Further, Lepeschkin [1922] states that increase of neutral salt concentration increases the rate of denaturation when the salt concentration is low and diminishes it when the salt concentration is high, leaving it unaltered at intermediate concentrations. He is of the opinion that the effects produced by the salts are to some extent specific.

It will be apparent from the above that no little confusion exists in the literature on this point, on account of the lack of distinction between the effect of salts in the cold, an action which they exert *per se* and their influence as external factors on the heat denaturation of proteins, which latter seems to be a reaction between the protein, the ions of water, and the water of hydration of the protein.

In the present work the effect of varying concentrations of ammonium sulphate, sodium chloride and sodium sulphate on the velocity of the heat denaturation of oxyhaemoglobin has been studied.

Experimental.

The solutions used were "neutral," that is to say the p_H as determined by use of the quinhydrone electrode at 37° was 6·76, at which point the concentration of hydrogen ion is equal to that of hydroxyl ion. The solutions contained various amounts of neutral salts.

Addition of neutral salt to the solution in general increases the hydrogen ion activity. It was decided to work at a constant hydrogen ion activity as this is the factor which it is desired to eliminate in the present work. As Sørensen, Heyrup and Lang [1921] have shown, the effect of salts on the p_H as measured by the hydrogen gas electrode and the quinhydrone electrode respectively is not the same. It was therefore decided to take as a standard of "neutrality" that solution which, at 37°, possesses a p_H value of 6·76 as measured by the hydrogen gas electrode, when correction was made to normal pressure of hydrogen over the solution[1]. In each case a small amount of acid or alkali was added to bring the p_H to 6·76.

[1] In order that the p_H of a solution might be determined *rapidly* the correction to be applied to a reading made by means of the quinhydrone electrode at any given salt concentration was determined.

To effect this the E.M.F. of a series of such cells as

Gold foil in presence of saturated quinhydrone	Buffer solution containing x% neutral salt	Platinum black saturated with hydrogen gas

The oxyhaemoglobin was prepared in the manner described in Part I. The same analytical technique was employed. As before the salts used were Merck's "puriss" thrice crystallised.

For the sake of brevity only the mean velocity constants are quoted. Each value recorded is the average of the mean value obtained from at least two concordant duplicate experiments.

SECTION 1. *The influence of varying ammonium sulphate concentrations on the velocity of the heat denaturation of oxyhaemoglobin in a solution of* p_H 6·76.

Table I. *Summarising table.*

70°

Concentration of ammonium sulphate (g. per 100 cc.)	(equivalents per litre)	$k_{uni} \times 10^4$
0·0067	0·00102	25·7
0·1	0·0152	27·0
1·0	0·152	28·0
5·0	0·758	45·5
6·0	0·910	49·0
7·5	1·137	54·0
10·0	1·52	36·7
15·0	2·274	14·0
20·0	3·032	5·7

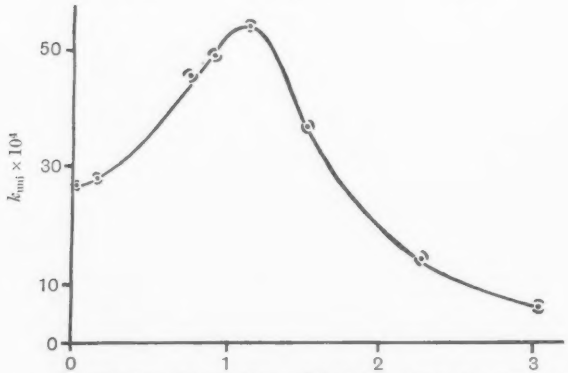

Fig. 1. Heat denaturation of oxyhaemoglobin at 70°. Variation of k_{uni} with concentration of ammonium sulphate.

was determined at 37°. The same solution was used throughout the cell, thus eliminating liquid/liquid potential difference.

The results of these experiments are as follows:

Neutral salt	$(NH_4)_2SO_4$	Na_2SO_4	NaCl
Concentration in normality	positive correction in millivolts		
0·0	0 0	0·0	0·0
0·5	1·9	1·8	1·6
1·0	3·6	3·6	3·2
2·0	7·2	7·1	6·4
3·0	—	10·5	—
4·0	14·1	—	12·1

From this table correction curves were drawn and values interpolated from the graphs were used in determining p_H by means of the quinhydrone electrode.

The data of Table I are plotted in Fig. 1. The effect shown here is noteworthy. Up to a concentration of 7·25 % of ammonium sulphate the velocity of denaturation is increased by increasing the amount of ammonium sulphate in the solution. From this point up to a concentration of 20 %, the velocity is steadily diminished, eventually falling below the initial value. This accounts for the contradictory statements referred to in the Introduction.

The ammonium sulphate seems to exert two opposing influences. One causes the velocity of denaturation to increase and the other causes it to decrease. The theoretical significance of this will be discussed later.

In the following table are given the results of a series of experiments analogous to those just cited.

Table II. *Summarising table.*

64°

Concentration of ammonium sulphate		
(g. per 100 cc.)	(equivalents per litre)	$k_{uni} \times 10^5$
0·0067	0·00102	34·1
0·1	0·0152	34·4
1·0	0·152	36·0
5·0	0·758	51·0
6·0	0·910	56·0
7·5	1·137	54·5
10·0	1·52	31·5
15·0	2·27	9·1
20·0	3·03	2·44

The data of Table II are plotted in Fig. 2.

These data again show the same effect as was noted in the case of the same reaction at 70° with the exception that, whereas the maximum occurred at a concentration of 7·25 % ammonium sulphate at 70°, it appears at a concentration of 6·7 % ammonium sulphate at 64°. This indicates that the action of the salt which tends to diminish the reaction rate is stronger at lower temperatures, so coming into play at lower concentrations.

SECTION 2. *The influence of varying concentrations of sodium chloride on the velocity of the heat denaturation of* 1 % *oxyhaemoglobin in a solution of* p_H 6·76 *at* 65°.

Table III. *Summarising table.*

Concentration of sodium chloride		
(g. per 100 cc.)	(equivalents per litre)	$k_{uni} \times 10^4$
0·054	0·00924	5·05
2·0	0·342	6·2
5·0	0·855	8·5
10·0	1·71	12·0
15·0	2·57	14·5
20·0	3·42	16·4

The data of Table III are plotted in Fig. 2. The results of this section show that with increasing concentration of sodium chloride up to 3·42 *M* (20·0 %) the velocity of denaturation rises steadily. There is a slight slowing off in the reaction rate with concentration but no actual fall of the velocity of denaturation. This means that the effect which caused an increase in the rate of reaction in the case of ammonium sulphate is now much stronger than the

other effect, namely that of reducing the reaction rate, which, it is evident, in this case does not predominate.

SECTION 3. *The influence of varying concentrations of sodium sulphate on the velocity of the heat denaturation at 65° of oxyhaemoglobin in solution of* p_H 6·76.

Table IV. *Summarising table.*

Concentration of sodium sulphate		
(g. per 100 cc.)	(equivalents per litre)	$k_{uni} \times 10^4$
0·015	0·000211	5·2
2·0	0·282	15·8
3·0	0·422	19·5
4·0	0·563	20·2
5·0	0·704	19·1
6 0	0·845	16·1
10·0	1·408	11·2
15·0	2·11	6·4
20·0	2·82	2·54

The data of this table are plotted in Fig. 2.

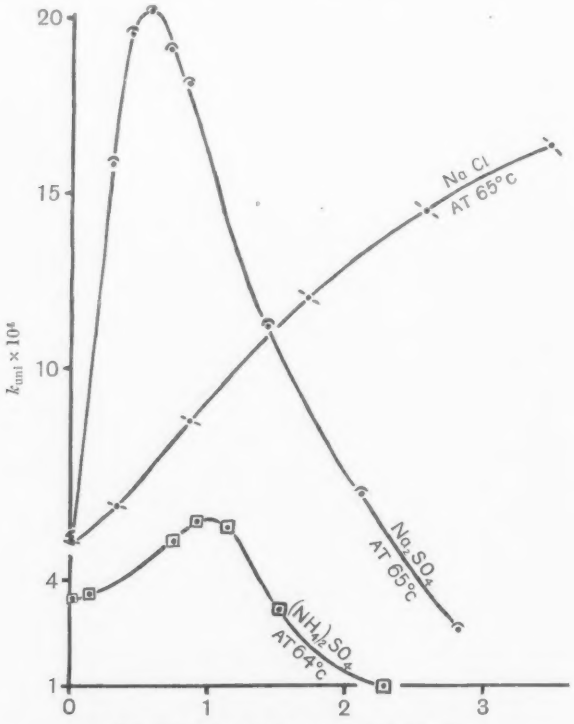

Fig. 2. Heat denaturation of oxyhaemoglobin in neutral solution. Variation of k_{uni} with salt concentration.

Here it is apparent that of the two opposing influences exerted by the sodium sulphate that which causes a reduction in the rate of denaturation sets in at a much lower concentration of this salt than in the case of ammonium sulphate. The relative effects of all the three salts studied are brought out in Fig. 2.

SECTION 4. *Heat denaturation of* 1 % *oxyhaemoglobin containing* 10 % *ammonium sulphate at* 68°.

In view of the theoretical considerations to follow it is convenient at this point to interpolate the following table which shows the variation of k_{uni} with p_H at 68° in presence of 10 % ammonium sulphate, although strictly this type of result belongs to Part I.

Table V. *Summarising table.*

p_H	$k_{uni} \times 10^3$
5·76	3·8
6·22	1·56
6·52	1·16
6·90	1·75
7·40	3·7

The data of Table V are plotted in Fig. 3, in which are also given the data of Table III, Part I, which shows the variation with p_H of the velocity of denaturation of 1 % oxyhaemoglobin containing 0·5 % ammonium sulphate. It will be seen that the two curves are similar save that the addition of the ammonium sulphate has displaced the curve p_H/k_{uni} downwards and to the acid side. The significance of this will be considered later.

DISCUSSION.

It is suggested that the action of the neutral salts depends on two distinct effects. It has been shown in Part I that with changing p_H the velocity of denaturation of oxyhaemoglobin in solutions containing relatively little salt (0·5 %) passes through a minimum when the activity of hydrogen ion is the same as that of hydroxyl ion, *i.e.* at the same p_H as that employed in the present instance. It is suggested that the first effect of the neutral salt (that which predominates in the lower concentrations of the sulphates and over the whole range in the case of sodium chloride) is to displace the minimum of the p_H/k_{uni} curve to one side. Thus the p_H 6·76 will no longer correspond to the minimum velocity but to some other, higher rate of reaction (cp. Fig. 3), thus causing the initial rise of velocity when the p_H is kept at 6·76 and the concentration of salt is increased.

The second action of the neutral salts is to depress the actual velocity of denaturation. This effect, predominating as it does in the higher concentrations of salt, is attributed to the dehydrating action of the salts. It is thought that this effect will only become apparent when the dehydrating action of the salts is sufficiently great to interfere with the hydration of the protein. This is

borne out by the relative magnitude of the influence of the various salts. In order of increasing power to depress the velocity they are

$$NaCl < (NH_4)_2SO_4 < Na_2SO_4.$$

This is also the order of increasing dehydrating power. Further, it will be noted in the case of the experiments with ammonium sulphate that this effect comes into play at lower concentrations of salt the lower the temperature. This is also in accord with the present view. In Part I it has been suggested that heat denaturation is a hydrolysis brought about by hydrogen and hydroxyl ions and likewise involving the water of hydration of the protein.

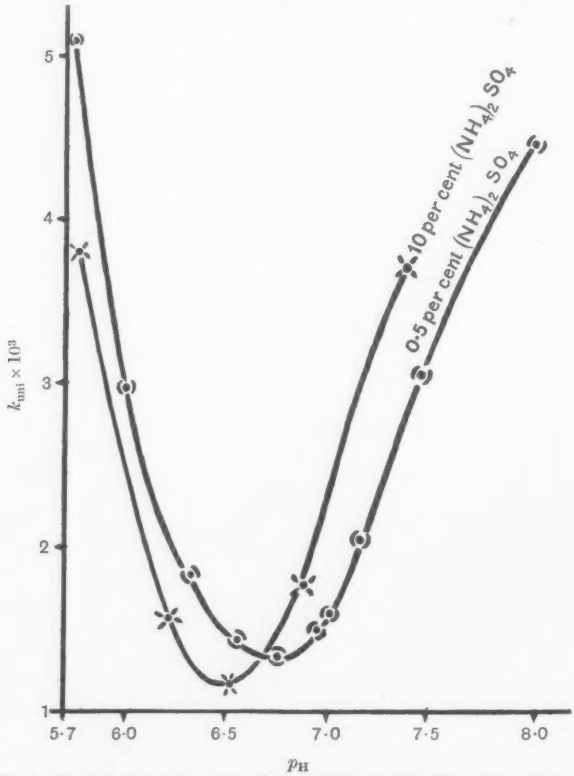

Fig. 3. Heat denaturation of oxyhaemoglobin at 68°. The effect of increase in the concentration of ammonium sulphate.

It will be seen that the present results are in entire accord with this hypothesis. Furthermore, they appear to be directly antagonistic to the alternative view, namely that denaturation is a chemical condensation between the carboxyl and amino groups of adjacent molecules, for were this the case the higher the concentration of neutral salt the greater would be the velocity of denaturation, owing to dehydration and consequent "exposure" of these groups.

The critical increment of the heat denaturation of oxyhaemoglobin.

In this case the critical increment has been calculated in two ways. First, the values of the velocity constants have been obtained by extrapolation to zero salt content on the graphs which show the variation of k_{uni} with concentration of ammonium sulphate. Secondly, the values given by the maxima on the same graphs have been used. It is argued, in the latter case, that the points chosen represent states in which the two opposing effects balance each other and hence the velocities to be used for a true comparison are those which correspond to this similar condition, and not the velocities at the *same* salt concentration.

In addition we have the value of the apparent critical increment as determined in Part I by utilising the velocity of the minimum point on the curves of p_H/k_{uni} (where the amount of ammonium sulphate present was small, namely 0·5 %).

Table VI. *The observed critical increment of the heat denaturation of oxyhaemoglobin.*

Source of data	Temp. in ° C.	k_{uni}	E in calories
Minima of p_H/k_{uni} curves	68·0	$1{\cdot}32 \times 10^{-3}$	77,500
	60·5	$1{\cdot}03 \times 10^{-4}$	
Maxima of Am_2SO_4/k_{uni} curves	70·0	$5{\cdot}4 \times 10^{-3}$	80,000
	64·0	$5{\cdot}68 \times 10^{-4}$	
Extrapolation to zero salt	70·0	$2{\cdot}68 \times 10^{-3}$	79,500
	64·0	$3{\cdot}42 \times 10^{-4}$	

It will be seen that the agreement is quite good. The significance of this critical increment has been discussed in Part I.

Summary.

The influence of varying concentrations of ammonium sulphate, sodium chloride and sodium sulphate on the velocity of denaturation of oxyhaemoglobin at constant hydrogen ion activity has been studied.

It is shown that the neutral salts appear to have a dual effect. The first, predominating in lower concentrations of salt, acts so as to displace the minimum of the curve of k_{uni} against p_H to one side. The second, which predominates in higher concentrations, acts so as to displace this same curve downwards.

It is shown that these results bear out an hypothesis advanced in Part I as to the mechanism of denaturation.

Values of the apparent critical increment of the heat denaturation of oxyhaemoglobin are calculated from the results obtained in presence of ammonium sulphate. These values are in good agreement with that previously found (Part I).

In conclusion the writer wishes to express his thanks to Prof. W. C. M. Lewis who suggested the subject of this investigation and whose helpful criticism has proved invaluable. In addition the author's gratitude is due to the Department of Scientific and Industrial Research for a grant which enabled this investigation to be carried out.

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CXXI. THE PECTIC SUBSTANCES OF PLANTS.

PART IV. THE PECTIC SUBSTANCES IN THE JUICE OF ORANGES.

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Our knowledge of the chemistry of the pectic substances of plants has made considerable strides in the last decade. The important work of Ehrlich [1917, 1924] on the hydrolysis of pectic substances indicated that the constituents of the molecule were arabinose, galactose and galacturonic acid. In these laboratories [Clayson, Norris and Schryver, 1921] a pure pectic substance was prepared, shown to have a definite chemical identity, and designated pectic acid. This substance was further examined by Nanji, Paton and Ling [1925] who found that the chemical properties of this substance were fully explained by a formula involving a six-membered ring, the sides of which represented arabinose, galactose and four molecules of galacturonic acid, the carboxyl groups of the latter being free. This substance may be regarded as the basal molecule to which members of the class of pectic substances may be referred. This formula received striking confirmation in the result of investigations on pectinogen, the precursor of pectic acid [Norris and Schryver, 1925]. It was found that the analyses of pectinogen corresponded with methylated derivatives of the basal molecule, and it was deduced that the pectic substance in the cell wall consisted chiefly of the trimethyl derivative of pectic acid, one carboxyl group being left intact; this is probably the point of attachment of the cellulose or hemicellulose with which pectinogen is associated.

The object of the present communication is to indicate the relationship between the pectic substance of the cell wall and that which is found in the juice of such a fruit as the orange.

It is generally held that the pectic substances of the juice are derived from the cell wall in the first instance, although the mechanism of the process is not as yet completely understood. It seems probable, however, that the acid in the juice is responsible for the scission of the pectic complex from the other constituents of the cell wall with which it is associated.

The reaction whereby the pectic substance is brought into solution is considerably less drastic than the chemical means employed to extract the product from the cell wall, and thus we should expect to find that this soluble pectic substance is highly methylated. The results of the present investigation

show that this is the case; the soluble product was precipitated with alcohol, could be redissolved in water and reprecipitated, and on analysis yielded both furfural and methoxyl corresponding almost exactly with those calculated for trimethylpectic acid.

Two points of interest are to be observed in connection with the preparation of this product.

Firstly, it is necessary to ensure that the juices come into intimate contact with the cell wall substance. If the juice is very carefully expressed in order to prevent the cells from being ruptured to any great extent, the amount of pectic substance present is small. If, however, the fruit is minced and filtered, the amount present is considerably increased, thus indicating that both cell wall and juice are intimately concerned in the production of the pectic substance.

Secondly, if after mincing in order to ensure the intimate association of cell wall and juice, the juice is allowed to stand for twenty-four hours or longer, a smooth jelly is formed, and this differs markedly from the soluble gel. The change is generally attributed to the action of enzymes and this view is supported by the observation that, if the juice is heated to 90–100° for an hour or two immediately after extraction, the formation of the smooth jelly is completely inhibited.

These two considerations, therefore, have some bearing on the method to be adopted in the preparation of the soluble gel. The oranges chosen were placed in boiling water for 2 hours; the peel was subsequently removed and the whole fruit minced. In this way enzyme action was stopped, and the intimate association of cell wall and juice and the maximum yield of soluble gel ensured.

The next step in the investigation was an attempt to identify the smooth jelly which is formed when the fruit juice is allowed to stand. Its properties were in direct contrast to those of the soluble gel. It was insoluble in water and contained no methoxyl groups. It could be dissolved in dilute ammonia and from this solution it was reprecipitated as a clear white gel by addition of excess of hydrochloric acid. These properties are characteristic of pectic acid and the yield of furfural obtained further supported this view.

Although the gels obtained were bulky, the actual weights finally obtained were very small; just sufficient for the analyses was obtained and further purification was therefore impossible. The ash content was not high, but a small quantity of nitrogen due to protein was present, and corrections were made in the analyses accordingly.

Attention was called in Part II of this series [Clayson, Norris and Schryver, 1921] to the fact that the analytical figures for pectic acid from oranges differed very slightly from those obtained for pectic acid from a variety of different sources. The same was true to a lesser extent in the case of apples. It is significant that these are the only cases in which any considerable quantity of pectic substance is to be found in the juice. Although at the present stage it

is not possible to explain this in detail, there seems little doubt that the activity of the acid and enzyme in the juice effects changes which might account for the slight differences in analysis cited above.

Experimental.

Preparation of the soluble gel.

About 30 Denia oranges, which were just ripe, were used for the preparations.

In order to stop the action of enzymes at the outset, the oranges were placed in boiling water and maintained at a temperature of 95–100° for 2 hours. The peel was then removed and the oranges were passed through a fine mechanical mincer. The moist mass was then filtered through muslin: this was somewhat slow, but by stirring the mass most of the liquid came through. The muslin bag was then placed in cloth and subjected to pressure. The extracts contained small quantities of cell wall material and were filtered on ordinary funnels; it was necessary to change the filter papers at intervals owing to clogging. Finally, however, the liquid was sufficiently free from solid particles to enable the first precipitation to be carried out. Accordingly it was thrown into two volumes of 95 % alcohol and the resulting gel allowed to stand overnight. The gel was filtered off with some difficulty through ordinary filters. At this stage the alcoholic filtrate was bright yellow and the gel itself was by no means colourless. The gel was redissolved in the minimum of distilled water, the solution filtered through paper pulp and again thrown into two volumes of alcohol. The gel had now lost most of its original colour, but further solution and precipitation were deemed necessary in spite of the fact that loss was involved.

After the second precipitation an almost colourless gel was obtained and this was then dried in graded strengths of alcohol for some days. After washing with ether and drying in a current of air, the product was placed in a vacuum desiccator over phosphorus pentoxide until it became constant in weight.

The analyses were carried out by the same methods as have been adopted in previous communications; furfural and methoxyl groups were estimated by the methods of Tollens and Zeisel respectively. The figures quoted were calculated on an ash-free basis. The calculated figure is based on accepted yields of furfural from the groups concerned [cf. Norris and Schryver, 1925], and shows the yields of furfural and methoxyl which may be obtained from trimethylpectic acid.

The results are indicated in the table below:

	Ash	Furfural	Methoxyl
	%	%	%
Found (i)	6·56	18·61	9·27
(ii)	—	19·12	8·44
(iii)	—	19·91	9·06
Average	—	19·21	8·92
Calculated		19·76	8·94

These results, therefore, correspond very closely with the figures calculated for trimethylpectic acid, and it is probable that the soluble gel consists almost entirely of this product. It resembles, in almost every respect, the pectinogen obtained by chemical means and described in Part III. It will be remembered that a hemicellulose was invariably found in association with pectinogen: owing to the small quantity of gel available in the present instance, it was impossible to effect a separation, and the question of the presence or otherwise of a hemicellulose in the soluble gel obtained from the juice must be held over until larger supplies have been prepared and examined.

Preparation of the insoluble gel.

The oranges were peeled and minced as already described. The minced mass was allowed to stand for 2 or 3 hours and then filtered through muslin. Filtration was slow, the juice in this instance being in contact with the cell wall for some hours. When filtration was complete, the cell wall substance was washed with a small quantity of distilled water and the filtered washings added to the main bulk of extract. Toluene and chloroform were added and the mixture allowed to stand for 3 days. At the end of this period, a considerable bulk of a brightly coloured jelly was formed and the residual liquid was poured off. The jelly was dissolved in ammonia, the mixture stirred mechanically for some hours and then filtered through paper pulp. A slight excess of concentrated hydrochloric acid was added, whereupon a flocculent gel was thrown down; the gel was thoroughly washed with many changes of distilled water and the process of solution and precipitation repeated. After another washing, the gel was almost colourless and was then dried as usual in graded strengths of alcohol, finally attaining constant weight after drying for a fortnight in a vacuum desiccator containing phosphorus pentoxide.

The results obtained on analysis are shown below:

		Ash %	Furfural %
Found	(i)	1·01	20·18
	(ii)	—	20·66
	(iii)	—	20·13
Average		—	20·32
Calculated		—	20·59

These results correspond very closely with those calculated for pectic acid; the properties of the gel are also similar and we may conclude that this product is identical with that obtained by chemical means from the cell wall.

It would seem, therefore, that the action of the enzyme on the pectic substance in the juice follows much the same course as was shown by the action of lime water on the soluble gel extracted from the cell wall (Part III). That is to say, the methylated pectic acid is gradually de-methylated, pectic acid being the final product. In the case of the pectinogen from the cell wall, however, it was shown that lime water effected, in addition, the scission of

a hemicellulose in association with the pectic substance. In the case of the product obtained from the juice, this has not yet been demonstrated and is left for future investigation when a larger supply of material is available.

Summary.

1. The pectic substances which occur naturally in the juice of fruit are essentially the same as those extracted from the cell wall by chemical means, as described in the earlier papers of this series.

2. They are derived from the cell wall by the action of the acids and enzymes in the juice; this view is supported by the observation that the quantity of pectic substance in the juice is considerably increased if the fruit is macerated before expression of the juice, thus allowing the juice to come into intimate contact with the cell wall.

3. The pectic substance in the juice corresponds with a trimethylated derivative of pectic acid similar to the pectinogen described in a former paper. This on standing is gradually converted into an insoluble gel unless the fruit is boiled before maceration and expression of the juice. The action is evidently due to the enzymes present in the juice. The insoluble gel has been identified as pectic acid.

4. Owing to the small quantity of material available, it has not yet been possible to determine whether a hemicellulose was present in association with the soluble gel. This was always found to be the case with preparations of pectinogen prepared from the cell wall by chemical means.

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CXXII. THE GRAVIMETRIC ESTIMATION OF BACTERIA AND YEAST.

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(*Received August 9th, 1926.*)

Since the micro-balance has reached its present state of perfection it has been possible to weigh to 0·01 mg. or even 0·001 mg. with accuracy and ease. It seemed likely that the micro-balance might provide a comparatively quick and easy method of estimating the growth in a bacterial or yeast culture where a knowledge of the total amount of cells (living plus dead) produced was required. In the present communication the technique which was found suitable for the estimation of *B. coli communis* growing in broth and also of *Saccharomyces cerevisiae* growing in glucose broth is given.

The estimation of *B. coli communis*.

The medium was a tryptic digest of caseinogen, described by Cole and Onslow [1916], the nitrogen content of which was equivalent to 4 mg. of nitrogen per cc. It was found, however, that when 10 cc. of this broth was run through a Pregl filtering tube a considerable amount of material was held by the asbestos of the filter. Moreover, the dry weight was not constant but varied between 0·15 and 0·35 mg. It was thought that this must be due to adsorption of the larger colloidal particles on the asbestos and to remove these the broth was subjected to a preliminary treatment with charcoal (Merck's). 500 cc. of the ordinary broth, after being adjusted to p_H 7·6, was mixed with 10 g. of charcoal, steamed for 1 hour and filtered. This process was repeated with a result that the medium was then almost colourless and gave a small and constant blank of about 0·12 mg. (incidentally, it was noticed that the medium gave no glyoxylic reaction).

By means of a pipette 10 cc. of the broth was measured into each of 50 test-tubes which had previously been thoroughly cleaned out with chromic acid and distilled water; these were then plugged and autoclaved. A cotton wool was chosen which did not tend to drop particles into the tubes.

Previous to inoculation the tubes were incubated for some hours so that the cells were sown into a medium of the same temperature (37°) at which the incubation was to proceed, thus reducing the lag due to a cold medium. Each tube was inoculated from an 11 hours' culture in broth identical with that used for the experiment. As the inoculation took a considerable time

an allowance of time had to be made for the later tubes, otherwise an appreciable error would be introduced into the estimation, which would be apparent on the steep part of the curve. At suitable intervals tubes were withdrawn and treated with 1 cc. of $N/10$ NaOH to coagulate the growth of bacteria and were well mixed. This method worked well with *B. coli* but for other organisms coagulation would probably occur at a different p_H and the method would need modification.

The contents of the tube were now filtered exactly as described by Pregl [1924] in the filters prepared for the filtration of silver chloride. The residue was washed with 200 cc. of 1 % acetic acid to remove any calcium phosphate, etc. which might be present, dried at 120°–125° by the usual method of Pregl and weighed.

Table I. *Dry weight of* B. coli *obtained from* 10 *cc. of medium.*

Time hours	Weight mg.	Weight due to medium mg.	Weight due to bacteria mg.	Average mg.
0	0·10 0·16 0·14 0·15 0·11 0·12 0·08	0·12 (average)	—	—
2	0·14	0·12	0·02	0·02
4	0·24 0·12	0·12	0·12 0·00	0·06
6	0·43 0·41 0·40	0·12	0·31 0·29 0·28	0·29
8	0·99 1·02 1·07	0·12	0·87 0·90 0·95	0·91
9	1·40 1·69	0·12	1·28 1·57	1·42
10	1·93 2·02	0·12	1·81 1·90	1·85
12 and later—average from Table II				1·91

The results showing the growth of *B. coli* are given in Table I and the average weight obtained at various hours is plotted in Fig. 1. It will be seen that the maximum error is most likely to occur when the growth is rising most rapidly and particular care must be taken in these estimations that no errors are introduced owing to slight differences in the time of sowing.

The remarkable feature of the results is the sharp flattening of the curve which occurs at the tenth hour (which has been confirmed in many experiments) and also the close agreement in the weight obtained at the twelfth hour and later (Table II). It will be noticed that in 13 estimations taken from different experiments there is an average difference from the mean of only 3·5 %. This close agreement has led us to place considerable confidence in the value of the method.

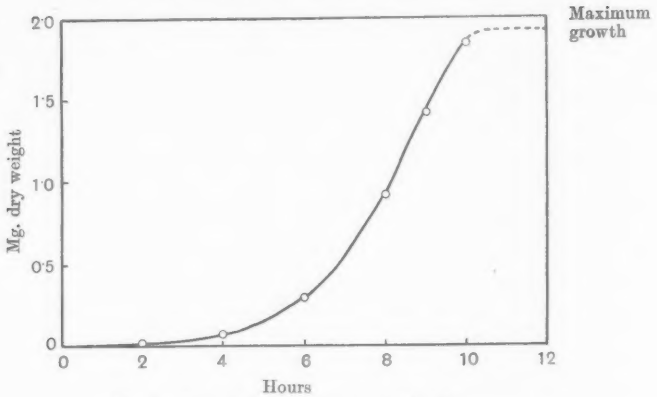

Fig. 1. Growth of *B. coli* on tryptic broth.

Table II. *Dry weight of maximum growth of* B. coli (12 *hours and after*).

No. of experiment	Weight of bacteria mg.	Difference from average %
1	1·87	− 2
2	2·04	+ 6
3	2·11	+10
4	1·90	− 0
5	1·83	− 4
6	2·06	+ 7
7	1·84	− 3
8	1·88	− 1
9	1·86	− 2
10	1·83	− 4
11	1·87	− 2
12	1·90	− 0
13	1·81	− 5
Average	1·91	3·5 %

THE ESTIMATION OF *SACCHAROMYCES CEREVISIAE* (NATIONAL TYPE COLLECTION 815).

The procedure adopted was very similar to that given above, but owing to the greater weight and size of the cells the estimation was considerably easier. The medium was a tryptic digest of one-quarter the strength used in the previous experiment and containing 1·5 % of glucose, the p_H being adjusted to 6·0. Incubation was carried out at 23°.

The inoculation was from a 48 hours' culture of yeast on the same medium; 0·2 cc. of the inoculating culture was introduced into each tube by means of a sterile graduated pipette. Unlike *B. coli* the weight of the inoculating cells was not negligible but amounted to 0·09 mg. It was found that this figure agreed remarkably well with that obtained in the experiment which followed where after 48 hours a weight of 4·01 mg. (Table III) of yeast was present in 10 cc. of medium. This gives a value of 0·08 mg. for 0·2 cc. corresponding with the 0·09 mg. actually found immediately after inoculation.

Table III. *Dry weight of yeast* (Saccharomyces cerevisiae) *obtained from* 10 *cc. of medium.*

Time hours	Weight mg.	Weight due to medium mg.	Weight due to yeast mg.	Average mg.
0 (medium)	0·08	0·10	—	—
	0·10	(average)		
	0·09			
	0·07			
	0·11			
	0·14			
0 (after sowing)	0·19	0·10	0·09	0·09
	0·20		0·10	
	0·18		0·08	
24	1·18	0·10	1·08	0·99
	1·00		0·90	
	1·08		0·98	
31	2·10	0·10	2·00	2·13
	2·26		2·16	
	2·33		2·23	
48	4·10	0·10	4·00	4·01
	4·12		4·02	
73	5·88	0·10	5·78	5·93
	6·19		6·09	
80	6·09	0·10	5·99	6·02
	6·16		6·06	
96	6·25	0·10	6·15	6·15
	6·25		6·15	
100	6·21	0·10	6·11	6·17
	6·34		6·24	

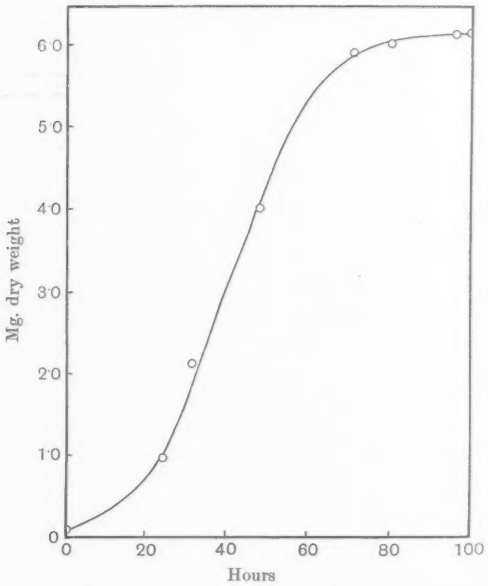

Fig. 2. Growth of *Saccharomyces cerevisiae* on glucose (1·5 %) tryptic broth.

The only notable difference in technique was that no coagulating treatment was necessary, because of the large size of the cells, and 200 cc. of water was used for washing in place of the acetic acid. Moreover, because of the much slower growth samples were taken off at longer intervals.

The results are given in Table III and the averages at the various hours have been plotted in Fig. 2.

In order to ascertain whether all the cells had been held back by the filtering asbestos one filtration was carried out with sterile precautions; 1 cc. of the filtrate was then withdrawn by means of a sterile pipette and inoculated into a test-tube of fresh medium and incubated. No growth took place.

One of us (H. I. C.) was in receipt of a personal grant from the Department of Scientific and Industrial Research and the other (M. S.) held a grant from the Medical Research Council. We also have to acknowledge a grant from the Royal Society and would like to thank Sir F. Hopkins for the interest he has shown in this work.

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CXXIII. THE ESTIMATION OF IODINE IN FOODSTUFFS AND BODY FLUIDS.

By ISABELLA LEITCH and JOHN McASKILL HENDERSON.

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(*Received August 11th, 1926.*)

The study of iodine metabolism has been retarded by the technical difficulty of estimating accurately the small amounts of iodine which occur in foodstuffs and in tissues and body fluids.

Kendall's method [1914, 1920], which is that most widely used at the present time, is admirably suited for the analysis of thyroid tissue, which contains roughly from 0·01 g. % to 0·5 g. % of iodine, and, as modified by Kelly and Husband [1924], it is in routine use at this Institute for that purpose. The method has, however, a lower limit of detectable iodine of 5γ ($\gamma = 0{\cdot}000001$ g.). For the estimation of iodine in human blood, therefore, Kendall required 100 cc., since the content is only 13γ %. The method is accordingly of little use for routine clinical purposes. The iodine content of milk is still lower than that of blood, and Forbes [1916], using Kendall's method, failed to detect iodine in any of 18 samples of milk.

Taking up the problem at the suggestion of the Swiss Goitre Commission, Fellenberg [1923, 1924] described a more delicate method, of which the best account is given by Veil and Sturm [1925] who have applied it to clinical work on blood. This method has a lower limit of detectable iodine of $0{\cdot}1\gamma$. Thus the iodine content of milk can be conveniently estimated in 10 cc., and that of blood in even less.

Fellenberg's method involves both a colorimetric and a titrimetric estimation. The material is ashed with potassium hydroxide and the ash extracted with alcohol. From this extract the iodine is liberated by sulphuric acid plus potassium nitrite. The liberated iodine is taken up in a drop of chloroform and estimated colorimetrically. Thereafter the iodine is oxidised to iodate by chlorine water and the solution evaporated to about 2 cc. The "iodide plus iodate" reaction is used as in Kendall's method and the liberated iodine titrated with $N/500$ thiosulphate, using starch as indicator.

With regard to the relative accuracy of the two estimations, it is claimed that the colorimetric is more accurate at the lowest levels. But the amount estimated by titration is six times the amount originally present and estimated colorimetrically. Hence, to be less accurate than the colorimetric estimation, the error in titration would have to be more than six times as great as the error

in the colorimetric estimation. This did not appear at all likely, and therefore, since the colorimetric estimation involves additional apparatus and a great deal more work, we decided to investigate the accuracy of the titrimetric estimation at the lowest levels, with a view to the possible elimination of the colorimetric part of the method.

We have made the following alterations in the procedure described by Veil and Sturm.

(1) Nickel crucibles are used instead of iron in view of the catalytic effect of iron and the consequent risk of loss of iodine in ashing.

(2) The colorimetric estimation is omitted: the residue after evaporation of the alcoholic extract is glowed and transferred at once to a 50 cc. flask for titrimetric estimation.

(3) Since the titrimetric estimation does not require the liberation of iodine, potassium nitrite is omitted. $2N$ sulphuric acid is used for acidification. We consider that the end-point is better with sulphuric than with other acids tried, *e.g.* hydrochloric or phosphoric.

(4) We use bromine water in place of chlorine water used by Fellenberg and Veil and Sturm. It is much more easily prepared and there is no difficulty in removing the last traces by boiling. We found the addition of salicylic acid, as recommended by Kendall, unnecessary.

As regards the delicacy of the titration, we found it possible to detect the difference produced by 0·002 cc. $N/500$ thiosulphate. This corresponds to 0·08 γ actual iodine.

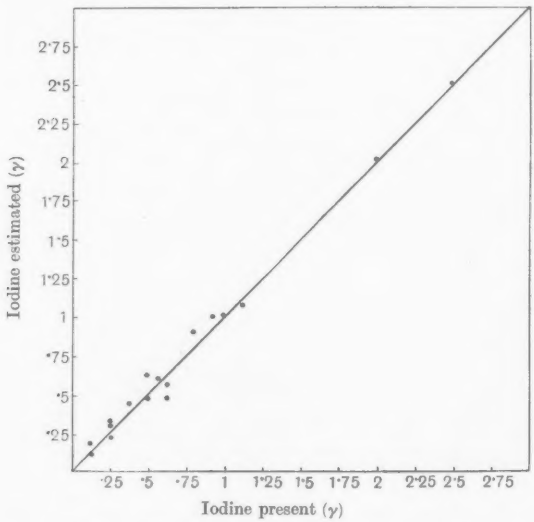

Fig. 1. Showing degree of accuracy of titration.

Accuracy of titration. The accompanying graph illustrates the relationship between actual and estimated iodine (after oxidation with bromine, etc.) when quantities varying from 0·125 to 2·5 γ are used. With quantities under 0·5 γ the error may be as much as 15 % of the total. It is less than 10 % between 0·5 γ and 1 γ, and above that it is negligible. We find that more than half of these titration errors with quantities below 1 γ are on the high side. This is due to the fact that an over-titration by as little as 0·001 (which does not give a detectable difference) means a positive error of 8 % at 0·5 γ. This error is, however, of little or no practical significance.

Recovery of iodine. To test the reliability of the method as a whole, we estimated known quantities of iodine added, as potassium iodide, to starch and to milk. Blanks of starch paste gave no iodine coloration when well ashed. A slight pink coloration occurred in two cases where the ashing was not good. Some representative figures for the recovery of added iodine are given in the following table:

Table I.

Substance used	Iodine present γ	Iodine added γ	Iodine estimated γ	Difference γ
1 g. starch	0	0·5	0·57	+0·07
1 ,,	0	0·5	0·53	+0·03
0·5 ,,	0	0·57	0·44	−0·13
1 ,,	0	1·0	0·9	−0·1
1 ,,	0	1·0	1·0	0
1 ,,	0	2·0	2·2	+0·2
1 ,,	0	2·0	2·3	+0·3
10 cc. goat's milk	1·2	0·5	1·9	+0·2
10 ,, ,,	1·2	0·5	1·7	0
10 cc. cow's milk	1·0	1·0	1·9	−0·1
10 ,, ,,	1·0	1·0	1·9	−0·1
10 ,, ,,	1·0	1·0	1·8	−0·2
10 cc. colostrum	0·5	1·0	1·25	−0·25

These figures indicate that the error in the method as a whole is about ± 10 % when the iodine is present in such minute quantities as 0·5 γ to 2 γ. Veil and Sturm [1925] state that there is an error of 10–15 % in the colorimetric method, and therefore, since the error in the titrimetric estimation is certainly not greater than this, even at the lowest levels, we have decided that we are justified in eliminating the colorimetric, and confining ourselves to the titrimetric estimation. The advantages gained are the saving of apparatus and time, and a very much simpler technique.

We have used the method with satisfactory results for work on blood, milk and pasture grasses. Papers on these subjects will follow.

Method.

The method as we use it is as follows.

Reagents and apparatus required.

(1) Potassium hydroxide sticks: iodine-free.

(2) Alcohol for extraction: 95 cc. absolute alcohol, 5 cc. distilled water.

(3) Methyl orange: 0·05 % *watery* solution.

(4) Sulphuric acid: approximately $2N$.

(5) Bromine water: to be prepared as required.

(6) Sodium thiosulphate: approximately $N/500$. This solution should be made up periodically from $N/10$ stock solution. $N/500$ thiosulphate does not keep well and must be standardised daily by means of potassium bi-iodate as described by Kendall [1920].

(7) A solution of potassium iodide made up just before the final titration. A small crystal is dissolved in about 20 cc. of water.

(8) Starch solution: to 50 cc. distilled water add a pinch of soluble starch. Boil for 1 to 2 minutes and cool. A fresh solution should be prepared every third day.

(9) Small chips of pumice stone, about the size of rice grains. These chips, after removing dust through a fine sieve, are boiled in dilute nitric acid, washed with distilled water, dried, and then strongly heated in the crucible furnace. After the estimation they are recovered. From time to time they are retreated and used over again.

(10) Davis combined crucible furnaces with Teclu burners. Vitreosil basins for the sand are most suitable.

(11) Nickel crucibles (6 cm. and 5 cm. in diameter). Nickel stirring rods.

(12) Flasks (50 cc. capacity).

(13) A serum pipette, 0·1 cc. capacity graduated in thousandths of a cc.

Procedure.

Ashing. A suitable quantity of the substance to be analysed is measured into a nickel crucible (6 cm. diam.), and 1 g. of potassium hydroxide stick added, dissolved in water in the case of dry substances. The contents of the crucible are stirred and then heated gently over a Bunsen burner till all bubbling ceases, and thereafter more strongly till no more fumes come off. Heating is continued on the furnace to a dark grey ash. The crucible must not be allowed to glow. When cool the ash is moistened with distilled water and carefully heated over a low flame till dry. The crucible is then returned to the crucible furnace and heated till no further change appears to be taking place (say 10 minutes). After cooling, about 5 cc. distilled water are added and the contents of the crucible filtered through a No. 40 Whatman ash-free filter-paper. (In all pouring operations it is necessary to vaselin the lip of the crucible with iodine-free vaselin.) The filter-paper is washed three times with about 5 cc. water. The filtrate, collected in a small beaker, is set aside. It should be clear, or at most, only very faintly yellow.

The filter-paper with its charred contents is returned to the nickel crucible, dried and then heated in the crucible furnace till a clean ash is obtained. The crucible is cooled, the filtrate returned to it and evaporated to dryness. This operation requires the greatest caution towards its completion, since spurting and crackling may cause loss. The crucible is again heated in the furnace for a minute or so, then cooled and about 3 cc. of water are added. This thick

solution is gently evaporated over a low Bunsen flame until a skin begins to form on the surface. Then the contents, after cooling, are ready for extraction.

Extraction. The ash is extracted three times with 3 cc. of the alcohol solution, the extract being filtered through a No. 40 Whatman paper into a 5 cm. crucible. The ash should form a smooth paste. If it is gritty one or two drops of water should be added, but excess should be avoided. After the third extraction the paste is poured on to the filter and the last of the alcohol allowed to drain. The filter paper is rejected, the funnel washed with a little distilled water and the filtrate evaporated to dryness on the water-bath.

The thin film of salt on the bottom of the crucible should be practically free from carbon. To get rid of any small particles which may be present, the bottom of the crucible is glowed gently for a few seconds, the salt film being just allowed to melt.

When the crucible has cooled the contents are dissolved in distilled water, and transferred to a 50 cc. flask.

Titration. Two drops of the methyl orange solution are added. The solution is rendered neutral by the addition, drop by drop, of $2N$ sulphuric acid. One drop is added after the neutral point. In all about three drops are generally required. Thereafter a few drops of bromine water are added. The red colour is immediately discharged. About 1 cc. bromine water should be added in excess of this, until the solution is distinctly yellow.

Three or four pieces of pumice stone are added and the solution is boiled over a medium flame, until the volume is reduced to between 1 and 2 cc., and then cooled. Two drops of the potassium iodide solution and two drops of the starch indicator are added and the titration carried out with $N/500$ thiosulphate from the 0·1 cc. pipette. By holding the flask at an angle it is possible by tapping the bottom of the flask with the point of the pipette to run in as little as 0·001 cc. at a time. If due precautions have been taken, the end-point is perfectly definite and after a little practice may be determined to about 0·002 cc.

Calculation: $\frac{\text{Titration figure} \times \text{iodine equivalent of thiosulphate}}{6}$ gives the amount of iodine in the sample.

SUMMARY.

Fellenberg's method of estimating iodine is modified by the elimination of the colorimetric part and concentration on the titrimetric estimation. In this form the method is much simpler and has been found suitable for routine work on blood, milk and pastures.

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CXXIV. OXIDISING ENZYMES IN THE PEEL OF *CITRUS* FRUITS.

By STANLEY GORDON WILLIMOTT and FRANK WOKES.

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(*Received August 14th, 1926.*)

In previous papers [Willimott and Wokes, 1925, 1, 2], the view was advanced that an important factor in the production of vitamins in *Citrus* fruits is photosynthetic action in the outer pigmented portion of the peel. Following up this idea, it was thought worth while to investigate the occurrence of oxidising enzymes in the outer pigmented portion, or flavedo, as compared with that of the inner colourless portion, or albedo, of the peels of different species of *Citrus* fruits. By so doing it was possible to ascertain whether these enzymes are located principally in the flavedo, or whether they are distributed evenly throughout the peel. It was also thought useful in the case of the lemon to examine the peel of fruit at different stages of growth, and to correlate the findings with the p_H of the tissues, with the object of obtaining data which might assist further investigations on the production of vitamins in the peel during the growth of the fruit.

Experimental Methods.

The fruits studied were the orange (bitter and sweet), the lemon and the grape fruit. The fruit was first well washed with cold water, then dried and the flavedo removed with a sharp knife, a small portion of the albedo being taken with it in order to rupture the least possible number of oil glands, thus avoiding as far as possible contamination of the albedo with products of the flavedo. The flavedo was freed from adherent albedo, and cut up into small pieces. The albedo left on the fruit was then removed, care being taken not to break through the peel with liberation of the pulp, and this was also cut up into pieces for experiment. In the case of the water and alcohol extracts, the minced tissue was thoroughly triturated with clean sand to ensure liberation of the juices, and then filtered, the filtrate being examined for oxygenase and for peroxidase and for aromatic catechol compounds by Onslow's method [1920, 1]. In testing for oxidising enzymes divergent results may be obtained according as tests are made on the fresh tissue or upon extracts of the tissue. The material was therefore tested both ways as recommended by Onslow [1920, 2]. The 10 % guaiacum tincture had previously been boiled with Merck's charcoal to remove peroxides. The catechol solution

had been tested with starch, iodide, and acetic acid and found free from peroxides. Whenever positive results were obtained with the extract of enzymes, a control experiment was done with a boiled extract which always gave a negative result.

In estimating the p_H of the lemon tissues, grinding with sand was found to produce an extract in which it was difficult to reach a sharp null point electrometrically; the tissue was therefore pounded with about an equal quantity of distilled water, and the resulting extract strained through washed cotton wool. The p_H of this strained liquid was estimated colorimetrically and also electrometrically, using a quinhydrone electrode.

The immature lemons used in this investigation had been specially picked for us in the San Dimas orchards by Mr C. P. Wilson, Chief Chemist of the Research Department of the California Fruit Growers Exchange. They were put immediately into cold storage, in which they remained until their arrival in England, where they were at once examined. It was observed that a number of these immature lemons, which after arrival in England had been put into a warm room while being examined botanically, began to develop a yellow colour within a week or so, whereas others kept in the ice chest retained their original green colour. This would seem to indicate that the precautions observed in storage conditions during transit had prevented any serious change in the fruit from the time it was picked off the tree in California to the time it was examined in England.

In recording the results on the immature lemons, as the exact age of each lemon was not known, they were arranged in order of weight. When these results were being plotted, a correction for the irregular growth of the fruit was required. By courtesy of Dr E. T. Bartholomew, of the University of California Graduate School of Tropical Agriculture, who has recently undertaken a comprehensive study of the growth rate of Californian lemons [1923, 1926], we were able to obtain data forming a suitable basis for this correction, which was applied in plotting the curves of p_H values at different ages.

All the other fruit used was fully grown, in ripe and perfect condition, and was purchased in the open market.

Results.

As noted by Onslow [1920, 2], the results of tests for oxidising enzymes may vary according to the ripeness of the fruit used. Thus, one sample of a given fruit may give a strong positive reaction with a certain reagent, while another sample of the same fruit tested similarly gives much weaker results. By careful selection of the fruit we were able independently to obtain results in agreement with each other in a large number of observations, which are summarised in Table I. It will be seen that we found a peroxidase in both flavedo and albedo of the four kinds of fruit tested, but no reaction could be obtained for oxygenase or for catechol aromatic compounds. This agrees with

Table I. *Reactions for oxidising enzymes given by peels of ripe* Citrus *fruits.*

	Bitter orange (*Citrus vulgaris*)		Sweet orange (*Citrus aurantium*)		Lemon (*Citrus medica* var. *Limonum*)		Grape fruit (*Citrus decumana*)	
Flavedo (mm.)	2		1		1		2	
Albedo (mm.)	5		2		2		6	
Browns on injury	—		—		—		—	
	Flavedo	Albedo	Flavedo	Albedo	Flavedo	Albedo	Flavedo	Albedo
A. Reactions of tissues								
Guaiacum	−	−	−	−	−	−	−	−
Guaiacum + H_2O_2	+	+	+	+	+	+	++	±
Benzidine + H_2O_2	+	+	+	+	+	+	++	+
α-Naphthol + H_2O_2	+	±	+	+	+	+	++	+
B. Reactions of water extract								
Guaiacum	−	−	−	−	−	−	−	−
Guaiacum + H_2O_2	−	−	±	−	−	−	+	−
Benzidine + H_2O_2	+	−	+	±	−	−	+	±±
α-Naphthol + H_2O_2	+	−	−	−	+	±	++	+
C. Reactions of extract of enzymes								
Guaiacum	−	−	−	−	−	−	−	−
Guaiacum + H_2O_2	−	−	−	−	+	±	+	±
Benzidine + H_2O_2	−	−	+	+	+	−	++	−
α-Naphthol + H_2O_2	−	−	±	−	±	−	+	+
Guaiacum + catechol	−	−	−	−	−	−	−	−
D. Reactions of aromatic extract								
$FeCl_3 + Na_2CO_3$	−	−	−	−	−	−	−	−

the results obtained by Onslow on the whole peel of the orange and lemon. Tests applied direct to the tissues showed practically no difference in peroxidase content of flavedo as compared with albedo in the case of the orange and the lemon, but in the grape fruit there is a markedly higher concentration in the flavedo. Tests applied to the extracts showed that the enzyme can be extracted from the flavedo much more readily than from the albedo. To avoid the inhibitory effect of tannins on oxidising enzymes, the extracts were treated with strong solution (about 25 %) of gelatin, filtered and tests applied to the filtrate. To ensure that the negative results obtained with extracts of the albedo were not due to adsorption of the enzyme by the spongy tissue, fragments of the alcohol-extracted tissue were added to the test-tubes containing negative tests, which in the case of the grape fruit produced a positive result.

A series of immature lemons of weights ranging from 1 g. to nearly full-grown was also examined, and the same result obtained, *i.e.* strong positive reactions for peroxidase in both flavedo and albedo, and negative reactions for oxygenase and aromatic catechol compound. In the smallest lemons (less than 5 g.) the freshly cut tissue gradually discoloured, but did not give the typical brown.

The p_H of the tissues in lemons of different stages of growth has been plotted in Fig. 1. The continuous line represents the average p_H of the peel at different ages, and the broken line the p_H of the juice. Little difference could be detected in p_H between flavedo and albedo at the same age. It will

be seen that both the peel and the juice gradually become more acid as the fruit matures until about four months old, when each reaches a fairly constant level. In the smallest lemons (less than 1 g.) the p_H of all the tissues would appear to be the same (about 5·8), but as there was insufficient material for electrometric determinations, and only colorimetric estimations could be made, these sections of the curves have not been completed. It is interesting to notice that the rapid increase of acidity which takes place between the ages of one and three months corresponds closely with the commencement of growth. Up to the age of one month the lemon increases very little in size. It then increases almost in geometric ratio up to about two months old, when it reaches a rate of growth which remains constant until the age of eight or nine months [Bartholomew, 1926].

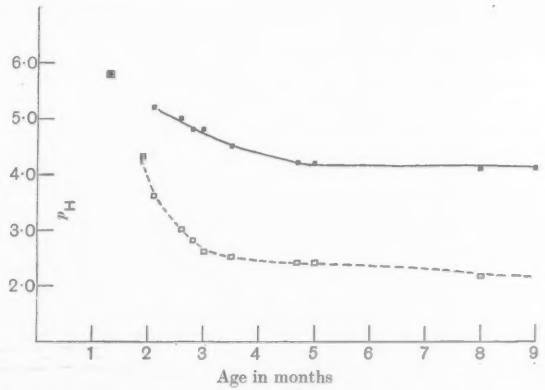

Fig. 1. Reaction of peel and of juice of Californian lemons at different ages.

SUMMARY.

The localisation of oxidising enzymes in the flavedo as compared with the albedo has been studied in the orange (bitter and sweet), the lemon and the grape fruit. Peroxidase only could be found, and appeared to be fairly uniformly distributed throughout the peel. The concentration in the flavedo was a little higher than in the albedo, but quite insufficient to explain the preponderance of vitamin in the flavedo. It must be borne in mind, however, that the whole of the peel is traversed by an elaborate conducting system, which we have found by microscopic examination to be well marked at all stages of growth.

The reaction of both peel and juice is acid at all stages of growth. In mature lemons we have previously found the peel to contain considerable amounts of vitamin B but very little C, while it is known that the juice contains much C but little B. The data now given on the increase of acidity with growth of the fruit should make it possible to plan further experiments

on the vitamin content of the peel and juice of immature fruit, in order to trace possible relationships between the p_H of plant tissues and the nature of the vitamins found therein.

We are indebted to Sir F. G. Hopkins and to Prof. J. MacLean Thompson for their interest in the work, and to Dr T. Redman for assistance in estimating the p_H of the material.

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CXXV. THE VITAMIN C OF LEMON RIND.

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(*Received August 27th, 1926.*)

In continuation of our study of the vitamins in the peels of *Citrus* fruits, we have investigated the antiscorbutic potency of the flavedo of the lemon by feeding an alcoholic extract as the sole source of vitamin C. According to Hess and Unger [1918], and to Harden and Zilva [1918], vitamin C is soluble in alcohol of all strengths. The use of 90 % alcohol avoids the possibility of extracting from the peel the oxidising enzymes, which we have found it to contain in large amount.

Details of the method of preparing the extract are given in a previous communication [Willimott, 1926]. Estimation of the total solids in samples taken at intervals from the extraction mixture showed that at the end of 48 hours extraction was practically complete. However, it was continued for not less than 7 days, when the mixture was filtered, and 1 cc. of the filtrate was considered to contain the vitamin C from 0·5 g. of rind. Preliminary feeding experiments having shown that a large dose of this filtrate was necessary in order to obtain antiscorbutic action, and bearing in mind the danger that the ingestion of large quantities of alcohol might vitiate the experiments, we prepared a stronger extract. To each cc. of the 1 in 2 extract another 0·5 g. of fresh peel was added and extraction continued for another 7 days. The filtrate thus obtained was considered to be a 1 in 1 extract, each cc. containing the vitamin C from 1 g. of rind. The 1 in 2 extract had the s.g. 0·905 and contained 3·4 % total solids, and the 1 in 1 extract had s.g. 0·959 and contained 6·3 % total solids. To prepare 100 cc. of this 1 in 1 extract, 13 or 14 ripe Palermo lemons are required.

Of the two methods available for testing for vitamin C, the superiority of the preventive method over the curative has been emphasised by Chick and Hume [1917], who found recovery in the latter to be uncertain and to lead to confusion in the interpretation of the results. The preventive method was therefore adopted. The basal ration of two parts of bran to one of oats was supplemented by 50 cc. per head *per diem* of fresh milk autoclaved at 15 lb. pressure, for 1 hour, as recommended by Chick, Hume and Skelton [1918]. Milk thus prepared contains no vitamin C and is a supplement to the basal ration rich in vitamin A and in calcium.

Four groups, each containing four healthy young guinea-pigs, were arranged so as to show correspondence as far as possible in sex and weight.

The sexes were segregated. The groups were given the following doses of the 1 in 1 extract per head *per diem*:

Group I. 0·5 cc., equivalent to 0·5 g. rind.
Group II. 0·75 cc., equivalent to 0·75 g. rind.
Group III. 1·0 cc., equivalent to 1·0 g. rind.
Group IV (Control). None.

The doses were administered orally by means of a hypodermic syringe. Considerable difficulty was experienced in inducing the animals to take the necessary dosage; dilution with water did not appear to render the extract any more acceptable. Goss [1925], working on a concentrated lemon juice, encountered a similar difficulty.

Growth effects.

In Group IV (Controls) there was no growth after the first week. The first death occurred on the 19th day, and the remaining three animals died in 6 days, the maximum period of survival being 25 days. Autopsies in each case showed typical signs of severe scurvy.

In Group I (on 0·5 g.) there was no growth after the second week, and the maximum period of survival was 31 days. The group as a whole showed severe scurvy.

In Group II (on 0·75 g.) there was no growth after the third week, the maximum period of survival was 37 days, and scurvy was confirmed in each case.

In Group III (on 1·0 g.) there was no growth after the third week, the maximum period of survival was 39 days. One animal which died unexpectedly on the 21st day was found to have impaction of the intestine. The symptoms of scurvy were not yet obvious, and it was difficult to say what was the cause of death. Two of the others died later of scurvy, and the fourth animal of the group was chloroformed on the 30th day, when in an advanced scorbutic condition.

Towards the end of the experiment (on the 22nd day), when it could be seen that the animals were not receiving their full antiscorbutic requirement, an attempt was made to increase the dose. As mentioned above, we found considerable difficulty in inducing the animals to take large doses of alcoholic extract, and were not able to raise the dose above 1·25 cc., which failed to cure. We then took 100 cc. of the extract, and concentrated it rapidly *in vacuo* at a temperature below 35° until practically all the alcohol had been driven off. The aqueous residue was made up to 50 cc. with distilled water, yielding a solution of which 1 cc. was considered to contain the vitamin C from 2 g. of lemon rind. To the two surviving animals in the control group we were able to give 2·5 cc. doses of this solution, equivalent to 5 g. of rind. Unfortunately, it appeared that scurvy had advanced too far for a cure to be effected, both the animals dying in 3 days when only two or three doses had been administered.

From our experimental results it would seem that the flavedo of ripe lemons contains only small amounts of vitamin C. Hess and Unger [1918], working on whole orange peel (flavedo *and* albedo), found that guinea-pigs given 0·5 g. of dried orange peel developed scurvy after 50 days, and were able to cure two out of three of the scorbutic animals by substituting for the dried peel an equivalent quantity of fresh orange peel. From our own data regarding the moisture content of orange and lemon peels, we conclude that the amount of fresh orange peel equivalent to 0·5 g. of dried peel would be from 1·5 to 2·0 g. But in the Report of the Accessory Food Factors Committee [1924, p. 69], it is stated, on the basis of Hess and Unger's work, that the protective dose of fresh orange peel is 5 g. There appears to be some discrepancy. From our work we conclude that the protective dose of fresh lemon flavedo is larger than that found for fresh orange peel by Hess and Unger. If this is so, it confirms the view put forward by Morgan and Chaney [1924] that the peel of the orange contains a higher proportion of vitamins than the peel of the lemon.

We are indebted to Sir F. G. Hopkins for his interest in the work.

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CXXVI. THE EFFECT OF SUBSTRATE CONCENTRATION ON THE HYDROLYSIS OF STARCH BY THE AMYLASE OF GERMINATED BARLEY.

By GEORGE SHARP EADIE.

From the Biochemical Laboratory, Cambridge.

(*Received August 16th, 1926.*)

It has been frequently assumed in the theory of enzyme action that the enzyme forms a combination with the substrate which breaks up to yield the products of the reaction and the free enzyme again. Further assumptions, such as that the velocities of combination of enzyme and substrate, and of the decomposition of their compound, follow the law of mass action, permit the deduction of mathematical expressions which connect the velocity of the reaction as a whole, at least in the initial stages, with the concentration of enzyme and substrate. Such expressions have been derived by Van Slyke and Cullen [1914], Michaelis and Menten [1913], and Briggs and Haldane [1925], and have been used by many authors. They have been particularly successful in the case of invertase.

An attempt to apply this theory to the case of barley amylase and starch has been made by Sjöberg and Ericson [1924]. Their data, however, seemed too scanty to afford a convincing proof that this theory was applicable, and indeed seemed to indicate that the relation which held between the initial velocity and the substrate concentration was other than that predicted by this theory. Their experiments have therefore been amplified.

Methods.

For substrate Lintner's soluble starch was used. Sjöberg and Ericson have shown that the difference between the rates of action of the enzyme on amylopectin and amylose is very slight.

The enzyme was prepared by allowing barley to germinate for 4 days; it was then ground thoroughly, extracted with water for 48 hours and filtered. Before use the extract was usually dialysed for several days against either distilled water or the buffer solution to be used. The extract before dialysis contained, of course, a large amount of reducing substance.

For buffer, a solution of sodium acetate and acetic acid was used, 0·2 *M* in acetate, and of p_H 5·1 unless otherwise noted.

The reacting solutions were brought to 15° before mixing and the reaction was allowed to take place in a vessel immersed in a water-bath at that temperature.

Reducing power was estimated in 5 cc. samples by the micro-method of Shaffer and Hartmann. The results are calculated as glucose.

The velocity was determined in the first 10 minutes in order to confine the estimation to the linear portion of the velocity curve. Estimations were usually made 1, 5 and 10 minutes after adding the enzyme and the average of the velocities in 4 and 9 minutes calculated. This gives double weight to the first period and was done in order to minimise any error from a falling-off of velocity in the second period. As a rule, the velocities in the first and second periods were identical, but in some cases experimental error was evident, in others the reaction was apparently going more slowly as it proceeded.

The production of reducing sugar from starch is probably the result of a series of reactions; but, since during the experimental period the rate is linear, it is evident that it is always the same reaction that is being measured. We are, therefore, not confused by the possibility of several reactions being reflected in varying degrees in the results.

Results.

The relation between velocity and substrate concentration.

Several curves were obtained, and a typical one is given in Fig. 1. Inspection of the curve shows at once that it differs very definitely from that predicted by Michaelis or Willstätter. These authors prefer to plot the velocity against the logarithm of the substrate concentration, and obtain a curve which is formally identical with that of the dissociation-residue curve of a weak acid. If the velocity be plotted thus it will be seen that two approximately straight lines are produced. Of these one represents the rising part of the curve, the other the flat portion where the velocity is zero.

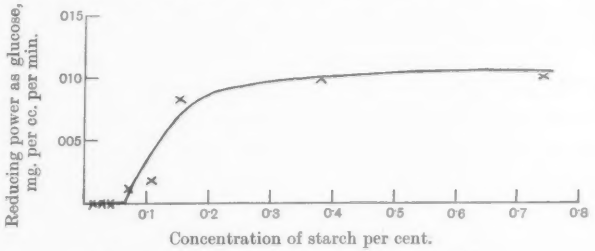

Fig. 1. With dialysed enzyme showing initial lag

Experiments in which undialysed enzyme was used gave similar results, as will be seen from Fig. 2.

In considering the significance of these results two possibilities present themselves. First, that the Michaelis type of curve would be found to apply

to systems of pure enzyme and substrate and is not found here because of the presence of impurities or on account of other experimental conditions. With regard to the last it will be shown that the curve is unaffected in shape by variations of p_H and temperature; nor will the absence of the proper salt concentration in the mixture explain the divergence. The criticism based on the presence of impurities cannot, of course, be disposed of until a pure enzyme preparation has been obtained, but various considerations render it improbable. The fact that experiments with undialysed enzyme show no essential difference in the shape of the curve points in this direction. Moreover, the considerable degree of purification of invertase achieved by Willstätter and by Euler has produced no essential alteration in the curve originally made by Michaelis with a relatively impure preparation.

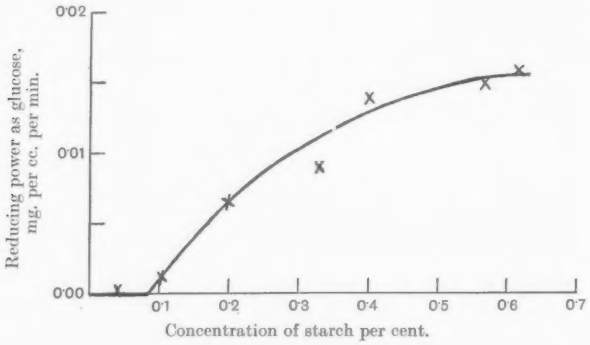

Fig. 2. With undialysed enzyme.

On the other hand, if this curve is really significant of the relation of velocity to substrate concentration, and I think that this must be assumed in the absence of proof to the contrary, we must conclude that the combination of enzyme and substrate follows in this case a rule other than the simple law of mass action from which the formulae referred to above have been derived; *i.e.* it is probably a heterogeneous equilibrium. This may mean some type of adsorption. While the curve has certain similarities to the adsorption curve, it differs decidedly near the zero point. If we take Freundlich's adsorption equation:

$$y = a.C^n \qquad \text{.....(1)}$$

where C is the concentration of adsorbed substance, y is the amount adsorbed, and a and n are constants, we may put C equal to the concentration of substrate (the concentration of the enzyme is constant), and y proportional to the rate, since the rate is presumably proportional to the amount of substrate combined, either "physically" or "chemically," with the enzyme. It is easy to show that this equation does not hold.

It is quite possible to obtain an empirical equation which will fit the results very exactly. Such an equation is

$$v = a + b \log C \qquad \text{.....(2)}$$

where v is the velocity, C is the concentration of substrate, and a and b are constants.

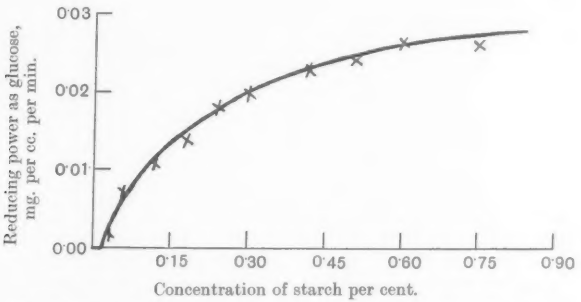

Fig. 3. The curve is plotted from equation (2): × experimental points.

This is illustrated in Fig. 3. The data for this have been chosen from another experiment in which more points were obtained for the rising part of the curve, although none for concentrations of starch so small as to give zero velocity (cf. Fig. 1). The curve is plotted from the equation

$$1000v = 20{\cdot}4 \log 10x - 10{\cdot}1.$$

To avoid decimals $1000v$ and $10x$ are used. Negative values of v are assumed to be without significance for the purpose of plotting the curve.

It may be noted in passing that a similar equation was used by Quastel and Whetham [1925] for the rate of reduction of methylene blue by *B. coli* in the presence of varying amounts of hydrogen donator.

I am unable to suggest any theoretical basis for this equation, but it forms a convenient method of comparing curves obtained under slightly different conditions.

The most interesting part of the curve is at the beginning. Until the concentration of starch rises above 0·03 %, the amount of hydrolysis in 10 minutes is nothing. In one or two experiments which were continued for 30 minutes no hydrolysis had occurred in this time. This limiting concentration appeared to vary only slightly with the enzyme preparation used; in some cases slight hydrolysis was seen at this concentration, but never with concentrations of starch less than this. Above this value, or sometimes a slightly greater one, there is a rapid increase in the rate of hydrolysis with increasing concentration of substrate, and the curve gradually falls off in a logarithmic manner.

The effect of neutral salts.

Because the activity of the enzyme is considerably decreased by dialysis, and in view of the well-known effect of sodium chloride on animal diastases, it was thought worth while to investigate this effect, although other workers have shown that plant diastase differs from animal in this respect.

The results are as follows:

Salt used	Concentration (normality)	Rate of hydrolysis
None	0·0	0·006
NaCl	0·064	0·007
,,	0·32	0·006
,,	1·62	0·009
KCl	0·038	0·006
K_2SO_4	0·154	0·007

The starch concentration was 0·385 %.

It will be seen that salts in large concentration have a slight effect. This, however, is insufficient to account for the loss of activity on dialysis.

The effect of enzyme concentration.

Two experiments were made using different enzyme preparations. The results of one of these are given in Fig. 4. It will be seen that the velocity is, within the limits of experimental error, directly proportional to the enzyme concentration. The concentration of starch was the same in all these experiments, viz. 0·385 %.

The other experiment which was made under the same conditions gave quite similar results with a thirty-fold variation in the relative concentration of enzyme.

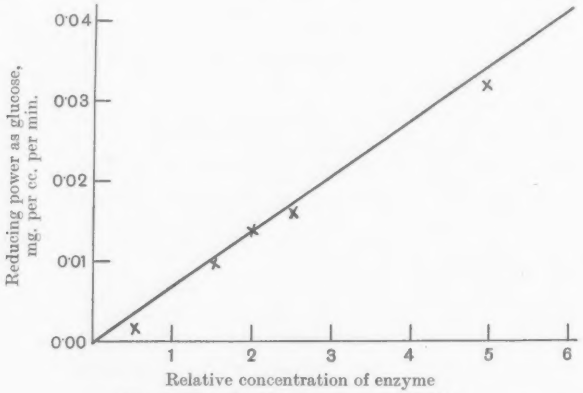

Fig. 4. The effect of enzyme concentration.

From these it is concluded that alteration in the amount of enzyme would have no effect on the general shape of the curve. This conclusion is further substantiated by the fact that, although several enzyme preparations of varying activity were used to determine the activity-substrate-concentration curve, it was always of the same shape.

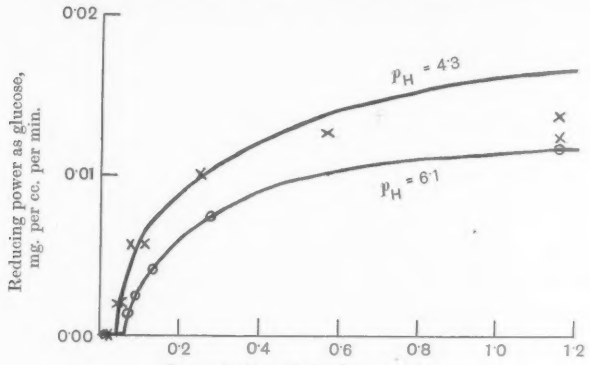

Fig 5. Showing the effect of p_H.

The effect of hydrogen ion concentration.

Curves were made for p_H 4·3 and 6·1. From Fig. 5 it will be seen that the effect is chiefly on the slope of the curve and only very slightly on the point where the zero value is reached. This is shown by the equations for these curves. The a of the equation affects the position of the point representing zero velocity, while the b (the coefficient of the log 100 C term) affects the slope.

At p_H 4·3 the equation is

$$1000v = 9{\cdot}9 \log 100C - 3{\cdot}9 \qquad \text{.....(3)}$$

and at p_H 6·1

$$1000v = 7{\cdot}7 \log 100C - 3{\cdot}8 \qquad \text{.....(4).}$$

The difference between 3·9 and 3·8 is possibly due to experimental error; at any rate it is not large enough to make a definite statement on this point possible.

The effect of temperature.

A curve was made at p_H 4·3 and a temperature of 5° which may be compared with the one made at p_H 4·3 and 15° and given in the preceding section.

The equation for the curve at 5° is:

$$1000v = 4{\cdot}2 \log 100C - 3{\cdot}5 \qquad \text{.....(5).}$$

From this and the corresponding Fig. 6 it can be seen that again the greatest effect is on the slope of the curve, but here there is a definite shift to the right.

It should be mentioned that the curves in this and the preceding section were made with all precautions to ensure that the activity of the enzyme was the same throughout. The enzyme preparation used had stood some months. At first its activity decreased rapidly from day to day, but by this time it had reached a practically stationary value. This is in agreement with the results of Sjöberg and Ericson. The curves were made on successive days and repetition of certain points on the curve showed that the enzyme had not changed.

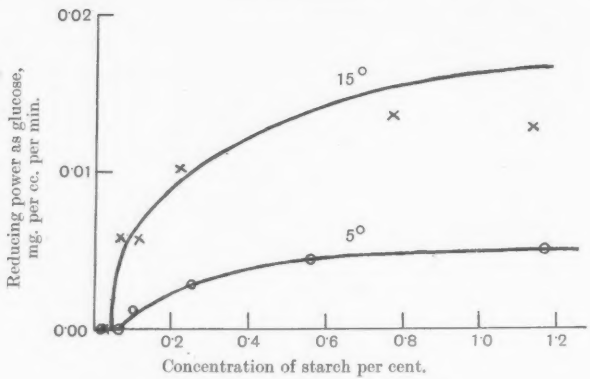

Fig. 6. Showing effect of temperature

The equations were deduced from the experimental data by the method of least squares.

It is interesting to calculate the temperature coefficients for varying starch concentrations. This may be done conveniently from equations (3) and (5). It is obvious from the figure that the use of the actual experimental data would lead to a similar result.

Starch concentration %	Q_{10}
0·0681	∞
0·0682	1000
0·0698	100
0·0716	50
0·0931	10
0·133	5
0·291	4
2·78	3

These figures show the great dependence of the temperature coefficient on the substrate concentration. In this case at least the temperature coefficient is meaningless without further details.

Glycogen as substrate.

An experiment was done using glycogen as substrate:

Glycogen concentration %	Rate
0·029	0·000
0·058	0·000
0·144	0·002
0·202	0·003
0·276	0·004
0·288	0·004
0·394	0·008
0·473	0·010
0·590	0·013
0·720	0·013
1·01	0·016
1·38	0·020

It will be seen that the affinity of the enzyme for glycogen is much less than for starch; the curve commences to rise only when a higher concentration of glycogen is used, and rises much less steeply. It is still rising, however, at the highest concentration used, while the curve for starch is practically horizontal in that region.

Discussion.

The bearing of these experiments on the velocity equation for the hydrolysis of starch may be pointed out. A great many equations have been proposed: they have been summarised by Kuhn in Oppenheimer's *Handbuch*.

The ordinary mono-molecular equation

$$dx/dt = k\,(a - x) \qquad \text{.....(6)}$$

implies that the initial rate ($x = 0$) is directly proportional to a, *i.e.* the initial concentration of substrate. Thus if, keeping everything else constant, one varies the substrate concentration, the relation found should be expressed by the formula

$$v = k.c \qquad \text{.....(7)},$$

where k is a constant, and v and c have the same significance as before. A comparison with equation (2) shows that this does not hold. It is therefore obvious that since (7) does not hold, (6) cannot represent the true course of the reaction, nor can any other equation which does not reduce, when $x = 0$, to (2) or an equivalent expression. None of the equations quoted by Kuhn apparently does so.

It is quite realised, however, that equation (2) is entirely empirical, and is not necessarily the only one which fits the facts, nor the one which may ultimately be found best to do so. On the other hand, any equation which seeks to embody the time-relations for the hydrolysis of starch must necessarily reduce under these conditions to this form or to an equivalent one.

Summary.

1. The relation of velocity of hydrolysis of starch by the diastase of germinated barley to the concentration of starch has been described and shown to differ from the relations previously described for other enzymes.

2. The effects on this of changes of salt concentration, of hydrogen ion concentration and of temperature are described.

3. A comparison is made with glycogen as substrate.

4. The temperature coefficient of the reaction is shown to vary from three to infinity according to the concentration of starch used.

My thanks are due to Sir F. G. Hopkins and to Mr J. B. S. Haldane for interest in and criticism of this work.

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CXXVII. NOTE ON THE ESTIMATION OF ACETONE BODIES BY THE VAN SLYKE METHOD.

By EDGAR CHARLES SMITH (*working for the Medical Research Council*).

From the Physiological Laboratories, Manchester University.

(*Received July 21st, 1926.*)

During an investigation on the effect of lactic acid on the production of acetone bodies from sodium butyrate in the perfused liver, a considerable discrepancy was observed between the values obtained by the Van Slyke and Fitz [1917] and Shaffer [1908] methods for the β-hydroxybutyric acid content of those samples of blood containing added lactic acid. Thus in one experiment, the total acetone produced in the first half-hour of perfusion, in which no lactic acid had been added (the concentration being 0·13 % as determined by the Clausen [1922] method), amounted to 110 mg. by the Van Slyke and 93 mg. by the Shaffer method. In the second half-hour, after addition of lactic acid to give a concentration of 0·28 %, the acetone formation as indicated by the Van Slyke method was 131 mg., whilst that by the Shaffer method was only 78 mg. When a known quantity of sodium β-hydroxybutyrate was determined by the two methods, the theoretical result was obtained in each case.

Pure lactic acid solutions of the same concentration as that of the blood, and samples of normal blood enriched with lactic acid, gave no precipitate when oxidised in the presence of mercuric sulphate, but a slight flocculent precipitate, similar to that described by Van Slyke and Fitz [1919], came down on cooling. It was concluded, therefore, that some derivative of either lactic or butyric acid must be responsible for the appearance of the extra precipitate. The effect of a number of substances which might affect the estimation was investigated, and the results may be classified as follows:

1. *Precipitate insoluble in the boiling reagent:* acetol, aldol, acetonylacetone, diacetyl.

2. *Precipitate soluble in the boiling reagent, coming down on cooling:* formaldehyde, acetaldehyde, lactic acid, pyruvic acid, formic acid (deep orange), glucose.

3. *No precipitate, even on cooling:* propionaldehyde, butyraldehyde, glycolaldehyde, glyoxylic acid, dihydroxyacetone, methylglyoxal, alanine, allantoin.

The substances in Group 1, if present in the blood after perfusion, would therefore cause a positive error in the estimation of acetone bodies by the Van Slyke method.

An examination of the precipitate from pure acetone showed that the acetone could be quantitatively recovered by distillation of the precipitate from 10 % HCl. The precipitate from the perfused blood, however, was found to yield only 50–60 % of the theoretical acetone, thus corresponding with the amount of acetone as determined by the Shaffer method. The distillate gave a reaction with ammoniacal sodium nitroprusside in saturated ammonium sulphate similar to that described by Speakman [1925] for pyruvic acid. A reinvestigation of the behaviour of lactic acid in the Van Slyke estimation showed that a quite small concentration of lactic acid, insufficient to give any precipitate when treated alone, caused a considerable increase in the amount of precipitate formed from β-hydroxybutyric acid; that the precipitate thus formed gave an amount of acetone corresponding with the amount of β-hydroxybutyric acid taken; and that the distillate gave the same reaction for pyruvic acid.

This means that the Van Slyke method gives inaccurate results in the presence of lactic acid owing to the oxidation of the latter to pyruvic acid, which, apparently, gives a mixed precipitate with acetone in concentrations very much lower than the minimal concentration required to form a precipitate in the absence of acetone. Concentrations of lactic acid of a magnitude sufficient to result in the precipitation of pyruvic acid are likely to occur quite frequently in blood, urine and tissue extracts. In blood used for liver perfusion, for example, the lactic acid concentration is normally of the order of 0·1 %, and the figure obtained by the Van Slyke method is always, to a greater or less extent, in excess of that obtained by the Shaffer method. An examination of the precipitate from diabetic urine treated in the manner described by Van Slyke showed that only 72 % of the theoretical acetone could be recovered by distillation from HCl, consequently it is likely that other substances than lactic acid may affect the precipitation in the same way, in addition to the substances in Group 1 which would certainly be precipitated. The Van Slyke method, although exceedingly rapid and convenient, cannot, therefore, be relied upon to give an accurate indication of the concentration of acetone bodies in biological material.

I wish to express my thanks to Prof. H. S. Raper for his constant advice and helpful criticism.

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CXXVIII. THE PIGMENTS OF BUTTERFLIES' WINGS.

II. OCCURRENCE OF THE PIGMENT OF *MELANARGIA GALATEA* IN *DACTYLIS GLOMERATA*.

By DAVID LANDSBOROUGH THOMSON
(1851 *Exhibition Senior Student*).
From the Biochemical Laboratory, Cambridge.

(*Received August 19th, 1926.*)

The author [1926] has previously described the isolation of a flavone-like pigment from the wings of the Marbled White butterfly, *Melanargia galatea*, and has suggested that it is probably derived from the grasses upon which the larva feeds. *Zea Mays* is the only member of the Gramineae from which flavones have been isolated [Sando, 1921; Remy, 1922], and the present research was undertaken in the hope of showing that they occurred also in one of the known food-plants of the larva. For this purpose common cock's-foot grass, *Dactylis glomerata*, was selected.

1 kg. of fresh *Dactylis* was chopped, dried in air, and soaked in cold water for 6 hours. It was then extracted with boiling 85 % alcohol for 12 hours, under reflux, and the extract concentrated to small volume and freed from alcohol. The aqueous suspension was then repeatedly shaken with ether, which removed the chlorophyll, lipochromes and uncombined flavones, the flavone glucosides and other pigments remaining in the aqueous layer.

The ethereal solution was washed with water and then shaken with dilute aqueous ammonia, into which the flavone passed. From the ammoniacal solution it was precipitated by acidification, dissolved in alcohol, and boiled with charcoal and filtered. From the filtrate the flavone was precipitated by diluting with water and removing the alcohol under reduced pressure. The precipitate was then redissolved in hot dilute alcohol and recrystallised four times until a constant melting point was obtained.

The aqueous solution containing the flavone glucosides was freed from ether and boiled for 24 hours with 5 % HCl. The liberated flavone was removed from the hydrolysate by shaking with ether, transferred thence to dilute ammonia and precipitated by acidification. The precipitate was washed with water and dissolved in dilute alcohol, and normal lead acetate solution added until precipitation ceased. The lead salt was washed with hot water and suspended in boiling alcohol, into which a stream of H_2S was passed for half

an hour. The alcoholic solution thus obtained, after removal of the lead sulphide, was filtered through asbestos, boiled with charcoal and filtered again, and finally precipitated and recrystallised from dilute alcohol as described above.

The isolated free flavone proved to be identical in all its properties with the flavone isolated from glucosidic combination. It is soluble in alkalis or concentrated mineral acids, with a bright yellow colour; very slightly soluble in water, even at boiling point, and sparingly soluble in ether; readily soluble in alcohol and acetic acid, and recrystallisable from these solvents. The alcoholic solution gives, with normal lead acetate, a deep yellow precipitate, with ferric chloride a dark brown coloration and green fluorescence, and with magnesium powder and HCl yields after some time a bright pink solution, from which a light green precipitate is obtained by adding excess of alkali. The melting-point was 251°. These properties correspond exactly with those described by the author [1926] for the pigment isolated from the wings of *Melanargia galatea*. The presumed identity of the two substances was confirmed by determining the melting-point of mixtures of the two substances, the results being 251°, 250·5°, 251°. It may be observed that, on account of the slight decomposition which takes place in the neighbourhood of the melting-point, it is not possible to obtain absolute agreement in a series of determinations on a single specimen.

The presence of free flavone in the plant was confirmed by its extraction from the fresh grass by a method which precluded hydrolysis during extraction. The fresh, chopped grass was extracted with boiling ether for 72 hours, the ethereal solution filtered hot, washed with water, and shaken with dilute aqueous ammonia, which removed from the ether a considerable quantity of free flavone (recognised by the colour tests described above).

It should be noted that the possible presence of smaller amounts of other flavones or flavone glucosides in *Dactylis glomerata* is not excluded.

Summary.

The grass *Dactylis glomerata* contains a flavone or flavonol which is present both free and in glucosidic combination, and which is identical with the pigment of the wings of the butterfly *Melanargia galatea*.

I am indebted to Miss M. S. Collingwood for invaluable help in the collection of the material, and to the Hon. Mrs Onslow and Sir F. G. Hopkins for their interest and advice.

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CXXIX. THE ACTION OF AMMONIA ON COMPLEMENT. THE FOURTH COMPONENT.

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During a study of the action of pancreatic extracts on complement [Wormall, Whitehead and Gordon, 1925], we became interested in the possible action of lipase, thinking that it might throw some light on the part played by fats and lipins in complement action. The chief difficulty was to obtain lipase preparations free from proteoclastic enzymes, for trypsin preparations have a destructive action on both the albumin and globulin fractions of serum. Eventually we used the enzyme preparation of Willstätter and Waldschmidt-Leitz [1923] which is stated to contain little or no trypsin and to have a powerful lipolytic activity. The preparation consists of a suspension of purified lipase in an ammoniacal phosphate solution containing glycerol, and the results obtained by its action on complement indicated that the destruction of a hitherto unknown factor had occurred. A control experiment, however, showed that the boiled enzyme solution had the same function, and by a process of elimination it was found that the active agent was really the ammonia. In effect, the addition of ammonia (or ammonium salts and alkali) to complement and incubation at 37° for 1–2 hours, destroyed the power to haemolyse sensitised red blood cells. The activity of the serum could be restored by the addition of complement which had been heated to 56° and which was by itself inactive. The ammonia thus appeared to have destroyed some relatively heat-stable component of complement.

The components of complement which are known up to the present, and which can be separated from each other, are (1) the globulin component (mid-piece), (2) the albumin component (end-piece)—both heat-labile, (3) a relatively heat-stable third component. The third component is usually associated almost entirely with the globulin fraction, but on occasion small quantities are found in the albumin fraction. By the action of CO_2 and dilute acids on complement two fractions may be obtained: the globulin fraction containing a globulin component and most or all of the third component, and an albumin fraction (containing an albumin component). The two fractions are inactive separately but together form a system practically as active as the original serum. By the action of yeast or of zymin, a preparation which is

stable and which gives more satisfactory results than yeast [Whitehead, Gordon and Wormall, 1925], the third component can be removed and the serum becomes inactive because it contains the albumin and globulin components but no heat-stable third component. This inactive zymin-treated serum can be reactivated by the addition of guinea-pig serum heated to 56° for half an hour or by the globulin fraction heated in the same way, these heated solutions containing none of the heat-labile protein constituents. Thus it has been shown that at least three components are necessary for the complete complement action.

Brooks [1920] considers that the protein constituents of serum play little part in the process and that there is only one active substance produced from a precursor which may resemble lecithin, but he states that the activity of this lytic substance may be dependent on the state of the serum colloids. In our opinion this view does not emphasise sufficiently the rôle played by the proteins, which, even if not actively concerned, act as carriers of the active substances. Any destruction of the protein components causes loss in activity as shown by the action of trypsin [Michaelis and Skwirsky, 1910; Wormall, Whitehead and Gordon, 1925], or by the destructive action of acids whenever the p_H falls below that of the isoelectric point of the globulin [Brooks, 1920]. Therefore since mid-piece and end-piece both contain heat-labile constituents which appear to possess the general properties of proteins, it is necessary to include these protein constituents when considering the nature of complement. Until more definite evidence is forthcoming, it is sufficient for ordinary purposes to regard them as globulin and albumin.

As has been stated, guinea-pig serum can be inactivated by treatment with zymin or small amounts of ammonia or ammonium salts and both the inactive sera so produced can be reactivated by the addition of serum which has been heated to 56° and which is itself devoid of complement power. Further examination, however, shows that the inactive sera are not identical. Firstly, the heat-stable factor which is needed to reactivate zymin-treated serum has been shown to be associated mainly with the mid-piece of complement; the factor which will reactivate ammonia-treated serum is present mainly in the end-piece fraction. Secondly, ammonia- and zymin-treated sera will mutually reactivate one another, showing that each is deficient in some factor which the other can supply. It follows that two relatively heat-stable components of complement are involved and we must therefore postulate four components in the original complement; globulin and albumin (mid-piece and end-piece) heat-labile components; the third component removed or inactivated by zymin, and the fourth component destroyed or inactivated by ammonia treatment. It does not of course necessarily follow that the four substances are separate and distinct in the original serum, for the process of splitting complement by dilute acids may divide some components which were present originally in combination with each other. Recombination of these separated components would presumably take place when complement was reconstituted by the

addition of end-piece to mid-piece. The significance of this distinction is made evident in the following paper.

. The CO_2 method of splitting complement does not always result in a clear-cut separation of the two heat-stable factors. It has already been shown that the third component is not always present in mid-piece only and similarly the fourth component, while present mainly in the end-piece, sometimes occurs in small quantities in the mid-piece. Consequently, while both mid- and end-pieces when fresh will often activate ammonia-treated serum, the amount of fourth component in mid-piece is so small that it is destroyed by heating for half an hour at 56°, so that of the heated fractions, only end-piece is effective.

After observing the main facts of inactivation by ammonia we were next concerned with attempts to elucidate the mechanism of the reaction. Other alkalis, such as caustic soda, were tried and it was found that their action was entirely different. Serum brought to the same p_H as the serum under treatment with ammonia, by addition of NaOH, and incubated in the same way, did not show a similar inactivation. In greater amounts or with a longer period of incubation there was a general destruction of complement which was not limited as with ammonia to inactivation of a heat-stable component. That is to say, an inactive serum produced by the action of NaOH could not be re-activated by the addition of any single component of fresh complement. The action of ammonia is therefore specific and not due simply to its alkalinity.

Ammonium salts were only effective when the p_H of the serum was 7 or over and the action was most pronounced at p_H 8–10. Any higher p_H (the reaction being adjusted with NaOH) resulted in a general destruction of the complement, identical with that observed when NaOH alone was used. Ammonia itself acted like other alkalis and destroyed the whole complement when added in amounts sufficient to raise the p_H beyond 10 or when the serum was incubated for long periods.

Other compounds containing NH_2 groups were then tried to see whether the action was due solely to that grouping. Amino-acids (glycine and alanine) and urea had no effect on complement in equivalent and in much larger amounts. Methylamine and ethylamine had the same effect as ammonia and it seems probable that the specific reaction is limited to ammonia and amines since the inactivation was not effected by neutral amino-compounds.

Time and temperature factors were then considered. By varying the conditions of temperature it was found that the time taken for inactivation was decreased with rise of temperature from 0° to 37°. At temperatures near freezing point the inactivation process was very slow and could be detected only after several hours. At room temperatures the velocity of the reaction was somewhat greater, but the best conditions were found to be the addition of 0·25 cc. of $N/6{\cdot}5$ NH_4OH to 1·0 cc. of guinea-pig serum and incubation for $1\frac{1}{2}$ hours at 37°. Under these conditions the action appeared to be specific towards the fourth component and remarkably regular, very rarely failing to give an inactive serum lacking only the fourth component.

Experimental.

The experiments were all carried out in duplicate at Leeds, where ox cells were used, and at Newcastle with sheep cells, and the results were identical at the two laboratories. The details of the haemolytic system were the same as previously used [Whitehead, Gordon and Wormall, 1925]. Guinea-pig serum was the source of complement and the results after incubation for 1 hour in an incubator or $\frac{1}{2}$ hour in a water-bath are indicated as follows:

+ + + +	complete haemolysis	+ + +	intermediate degrees
+ +	half haemolysis	+	,,
–	no haemolysis		

Preparation of mid-piece and end-piece.

This was effected as described before [Whitehead, Gordon and Wormall, 1925] and in all experiments the excess of CO_2 was removed from the end-piece by evacuation and both fractions were adjusted to p_H 7·5.

The inactivation of complement by ammonia.

The inactivation described here was first obtained by using preparations of lipase prepared by Willstätter's method, but by elimination it was found that ammonia was the agent concerned and therefore an equivalent amount of ammonia was used. To 1 cc. of guinea-pig serum 0·25 cc. of $N/6·5$ NH_4OH was added, the mixture shaken and incubated for $1\frac{1}{2}$ hours at 37°. The serum was then adjusted, neutralised with $N/10$ HCl to p_H 7·5 and diluted to 10 cc. with physiological saline. This liquid (A) had no complete complement action itself but haemolysed sensitised cells if end-piece, end-piece heated at 56° for $\frac{1}{2}$ hour, or heated guinea-pig serum was added.

Table I.

1·0 cc.	A	—	–
0·5 cc.	A	+ 0·5 cc. 0·9 % NaCl	–
0·5 cc.	A	+ 0·5 cc. mid-piece	– or +
0·5 cc.	A	+ 0·5 cc. heated mid-piece	–
0·5 cc.	A	+ 0·5 cc. end-piece	+ + + +
0·5 cc.	A	+ 0·5 cc. heated end-piece	+ + + +
0·5 cc.	A	+ 0·5 cc. heated complement	+ + + +

Solution A and all other solutions which had no complement action were tested for anticomplementary power. None of these solutions was anti-complementary and in addition, an amount of ammonium salts equivalent to or much greater than that present in solution A was likewise devoid of anti-complementary action. Thus the inactivity of A and similar solutions is due to the absence of some essential constituent or constituents.

Note. In the above and in all the experiments described in this paper the mid-piece and end-piece fractions were separately inactive but when mixed together they had a specific haemolytic action on sensitised cells almost as powerful as an equivalent amount of the original guinea-pig serum. The heated solutions were obtained by heating the fractions or the serum in a water-bath at 56° for 30 minutes.

Differences between sera inactivated by ammonia and by zymin (or yeast).

Guinea-pig serum was inactivated by ammonia as described above, and by zymin as described previously [Whitehead, Gordon and Wormall, 1925]. The neutralised solutions were both inactive but together were completely active. Each inactivated serum could be activated by the addition of heated serum, but, whereas the ammonia-treated serum was rendered active by the unheated or heated end-piece fraction, the zymin-treated serum was activated by the unheated or heated mid-piece fraction. Neither serum had any anti-complementary action when tested with varying amounts of fresh guinea-pig serum.

Table II.

A = Ammonia-inactivated serum. Z = Zymin-inactivated serum.

1·0 cc. A –	1·0 cc. Z –		A	Z
0·5 cc. inactivated serum (A or Z)		+ 0·5 cc. 0·9 % NaCl	–	–
,,	,,	+ 0·5 cc. mid-piece	–	+ + + +
,,	,,	+ 0·5 cc. heated mid-piece	–	+ + + +
,,	,,	+ 0·5 cc. end-piece	+ + + +	+
,,	,,	+ 0·5 cc. heated end-piece	+ + + +	–
,,	,,	+ 0·5 cc. heated guinea-pig serum	+ + + +	+ + + +

Strong confirmatory evidence that the two inactivated sera are deficient in different heat-stable factors has been obtained from experiments with heated guinea-pig serum and heated pig serum. Pig serum, which is extremely rich in the third component, contains much less of the fourth component, whilst guinea-pig serum is rich in both factors. Ammonia acting upon heated guinea-pig serum destroys the fourth component but not the third component, and the same amount of ammonia has no apparent action on the third component of heated pig serum.

2 cc. guinea-pig serum and 2 cc. pig serum heated at 56° for 30 minutes.

(*a*) 1 cc. of each serum was diluted to 10 cc. with 0·9 % NaCl.

(*b*) 1 cc. of each undiluted heated serum was treated with 0·25 cc. $N/6{\cdot}5$ NH_4OH, incubated at 37° for 2 hours, neutralised and diluted with 0·9 % NaCl to 10 cc.

Table III.

A and Z as in Table II.

			A	Z
0·5 cc. inactivated serum (A or Z)		+ 0·5 cc. heated guinea-pig serum (*a*)	+ + + +	+ + + +
,,	,,	+ 0·5 cc. NH_3-treated heated guinea-pig serum (*b*)	–	+ + + +
,,	,,	+ 0·5 cc. heated pig serum (*a*)	+	+ + + +
,,	,,	+ 0·5 cc. NH_3-treated heated pig serum (*b*)	–	+ + + +

Progress of the process of inactivation by ammonia and the influence of temperature.

Serum was treated with one-quarter of its volume of $N/6{\cdot}5$ NH_4OH and divided into three portions. One was kept in ice, one allowed to stand at room temperature, and the third incubated at 37°. Samples were withdrawn from each at the beginning of the experiment and at various intervals of time. The

samples were adjusted immediately to p_H 7·5 and diluted with saline so that they were equivalent to 1 : 10 complement. They were tested for action on sensitised cells with and without the addition of heated end-piece and heated serum. Heated or unheated mid-piece had no activating power, *i.e.* contained no fourth component.

Table IV.

Temp.			Time of incubation (hours) 0	½	1	2	3	4
37°	1·0 cc. NH_3-treated serum		++++	−	−	−	−	−
	0·5 cc. ,,	+0·5 cc. 0·9 % NaCl ...	++++	−	−	−	−	−
	0·5 cc. ,,	+0·5 cc. heated end-piece	++++	++++	++++	++	·+·	−
	0·5 cc. ,,	+0·5 cc. heated serum	++++	+++	+++	+	+	−
20°	1·0 cc. NH_3-treated serum		++++	++++	−	−	−	−
	0·5 cc. ,,	+0·5 cc. 0·9 % NaCl ...	++++	++	−	−	−	−
	0·5 cc. ,,	+0·5 cc. heated end-piece	++++	++++	++++	+++	+++	+++
	0·5 cc. ,,	+0·5 cc. heated serum ...	++++	++++	++++	+++	++	·++
0°	1·0 cc. NH_3-treated serum		++++	++++	++++	+	−	−
	0·5 cc. ,,	+0·5 cc. 0·9 % NaCl ...	++++	++++	+++	−	−	−
	0·5 cc. ,,	+0·5 cc. heated end-piece	++++	++++	++++	++++	++++	++++
	0·5 cc. ,,	+0·5 cc. heated serum ...	++++	++++	++++	++++	++++	++++

Specificity of the action of ammonia. Comparison with the action of sodium hydroxide.

(*a*) Serum was adjusted by means of *N*/10 HCl or *N*/10 NaOH to approximate p_H values ranging from 7 to 11. An amount of NH_4Cl equivalent to the NH_4OH used in the previous experiments was added and the solutions incubated at 37° for 1½ hours. The solutions were then neutralised and tested with the various fractions of complement as before. Controls were carried out with serum adjusted to the same p_H values but without NH_4Cl. The inactivation of fourth component was noticeable only in alkaline solutions and was most pronounced at p_H 8·5–10·0. Controls at the same reactions, without NH_4Cl showed no destruction of complement power. Above p_H 10·0, the serum with or without NH_4Cl had lost all haemolytic action and could not be reactivated by any heated or unheated complement fraction. Thus above this p_H the action appeared to be a general destruction of the whole of the complement, due to the alkali present.

Experiment. 1 cc. guinea-pig serum + *N*/10 HCl or *N*/10 NaOH to required p_H + 0·25 cc. *N*/6·5 NH_4Cl.

Control. 1 cc. guinea-pig serum + the same amount of HCl or NaOH + 0·25 cc. normal saline.

All solutions incubated at 37° for 1½ hours, adjusted to p_H 7·5 and diluted with normal saline to 10 cc.

Table V.

	p_H 7·0	7·5	8·0	8·5	9·0	10	11
Serum with NH_4Cl	7·0	7·5	8·0	8·5	9·0	10	11
1·0 cc. serum	++++	++++	++++	−	−	−	−
0·5 cc. serum + 0·5 cc. saline	++++	++++	++	−	−	−	−
,, +0·5 cc. heated end-piece ...	++++	++++	++++	++++	++++	++	−
,, +0·5 cc. heated serum (1 in 10)	++++	++++	++++	++++	++++	++	−
Serum without NH_4Cl	7·0	7·5	8·0	8·5	9·0	10	11
1·0 cc. serum	++++	++++	++++	++++	++++	−	−
0·5 cc. serum + 0·5 cc. saline	++++	++++	++++	++++	++++	−	−
,, +0·5 cc. heated end-piece ...	++++	++++	++++	++++	++++	−	−
,, +0·5 cc. heated serum ...	++++	++++	++++	++++	++++	−	−

Note. If the solutions with NH_4Cl at p_H 7·5 and 8·0 were left in the incubator for longer periods, destruction of the fourth factor took place, but this inactivation was very slow, especially at p_H 7·5.

(*b*) Serum was treated with varying amounts of NH_4OH and a similar series with equal amounts of NaOH. Incubation and other details were carried out as before. In no case did any specific inactivation of the fourth component occur with NaOH and where inactivation did occur it was due to general destruction of the whole of the complement. In relatively small amounts NH_4OH was quite effective in removing the fourth component whereas an equal amount of NaOH had no action.

Table VI. *Amounts of alkali to* 1 *cc. of serum.*

			0·25 cc. *N*/6·5 NH_4OH	0·25 cc. *N*/13 NH_4OH	0·25 cc. *N*/26 NH_4OH
1·0 cc. treated serum 1/10			−	−	+ + + +
0·5 cc.	,,	+0·5 cc. saline	−	−	+ +
0·5 cc.	,,	+0·5 cc. heated mid-piece	−	−	+ +
0·5 cc.	,,	+0·5 cc. heated end-piece	+ + + +	+ + + +	+ + + +
0·5 cc.	,,	+0·5 cc. heated serum	+ + +	+ + + +	+ + + +
			0·25 cc. *N*/6·5 NaOH	0·25 cc. *N*/13 NaOH	0·25 cc. *N*/26 NaOH
1·0 cc. treated serum 1/10			−	+ + + +	+ + + +
0·5 cc.	,,	+0·5 cc. saline	−	+ + + +	+ + + +
0·5 cc.	,,	+0·5 cc. heated mid-piece	−	+ + + +	+ + + +
0·5 cc.	,,	+0·5 cc. heated end-piece	−	+ + + +	+ + + +
0·5 cc.	,,	+0·5 cc. heated serum	−	+ + + +	+ + + +

General properties of the fourth component.

(*a*) This component is more stable at 56° than the two protein factors and in this respect resembles the third component. End-piece fraction if heated at 56° for ½ hour still contains the bulk of the fourth component although the albumin component has been destroyed. More prolonged heating slowly destroys the fourth component and after 1 hour at 56° there is little or none left in the solution. Like the third component, the fourth component is destroyed by heating for ½ hour at 66°.

(*b*) The fourth component is present mainly, if not entirely, in the end-piece and is therefore not precipitated by CO_2. It is non-dialysable since it is present in serum or end-piece which has been dialysed against water or saline in a parchment or collodion membrane for 24 hours. It is not removed from serum by treatment with zymin or yeast.

(*c*) It is destroyed when serum is made definitely acid or alkaline and incubated at 37° for 2 hours.

Summary.

(1) Small quantities of NH_3 have a destructive action on complement, removing a factor hitherto unrecorded. This component and the three other components previously recognised, must all be present for complete complement activity. Haemolysis of sensitised red cells only takes place therefore in the presence of two heat-labile protein components and two relatively heat-stable components.

(2) The fourth factor is relatively heat-stable and is associated with the albumin fraction. Ammonia-inactivated serum can thus be reactivated by the addition of heated serum or by heated or unheated albumin fraction.

(3) The fourth component is not identical with the third component which is removed by yeast or zymin, for the two components are associated with different fractions after the splitting of complement by dilute acids. Also, serum inactivated by zymin will reactivate ammonia-inactivated serum.

(4) The inactivation process is not immediate, but after a given time, depending on temperature and strength of ammonia, the fourth component is destroyed, leaving the other three components intact. At a later period the other components are destroyed owing to the alkalinity of the solution.

(5) Other alkalis have no similar specific action on the fourth component. Ammonium salts only act when the serum is previously adjusted to an alkaline p_H, the best reactions being above p_H 8·0 and below p_H 10·0. Thus free NH_3 is necessary for the inactivation process.

(6) The effect of temperature is very pronounced. The velocity at 37° is at least four times that at 0°.

(7) This new component is non-dialysable and not destroyed by heating at 56° for ½ hour, but it is readily destroyed at 66°. It is also destroyed by the action of dilute acids and alkalis at 37°.

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CXXX. CALCIUM AND COMPLEMENT ACTION.

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Liebermann [1921] has expressed the view that the active complement responsible for immune haemolysis is none other than the soaps normally occurring in the serum. He postulates that the general haemolytic properties of these compounds are suppressed by other constituents of the serum, proteins and calcium compounds, so that haemolysis results only in the presence of the sensitising substance. Thus this author claims to be able to prepare an artificial complement by adding to heat-inactivated rabbit serum a methyl alcoholic solution of calcium oleate, the mixture obtained being haemolytic towards sensitised cells but having no effect on unsensitised cells. Similar solutions have been prepared by Freund [1923], who used an aqueous solution of calcium oleate and the globulin fraction of bovine serum. Schmidt [1924], however, has been unable to repeat these results and states that the solutions obtained have no specific action on sensitised cells. Liebermann himself appears to have experienced difficulty in this respect and attributes it partly to the impossibility of obtaining suitable methyl alcohol. Although he maintains that his artificial complement will give satisfactory results when used instead of natural complement in the Wassermann reaction, he admits that some improvement in the technique of preparation is necessary before it can be used extensively in practice.

With the exception of this work on the preparation of artificial complement, there is little evidence available of the possible rôle played by calcium compounds in complement action. Various authors (Wright and MacCallum [1922] and Purdy and Walbum [1922]) have shown that calcium salts exert an anticomplementary action, even small amounts inhibiting haemolysis, and it is possible that the occasional anticomplementary action of certain fractions of complement may be due in part to the small amount of calcium salts present. The amount of calcium normally present in serum is not sufficient to exert any powerful anticomplementary action in the whole serum, but the amount may be sufficient for the calcium to have some active part in the complement reaction.

The inability of other workers (including the present authors) to prepare artificial complement by Liebermann's method may be due to some slight

differences in technique and the question of the validity of Liebermann's views appears to warrant investigation along other lines. If this view of the nature of complement is correct, then the artificial complement, besides having a specific haemolytic action on sensitised red cells, should be heat-labile, and CO_2 and dilute acids should separate it into two inactive fractions which together have full complement activity. The splitting of complement by CO_2 has not yet been explained on any other grounds than the precipitation of the globulin fraction and it is of interest, therefore, to consider what would happen if complement consisted principally of calcium salts of fatty acids associated with the serum colloids. The calcium in serum is only partially dialysable, and the non-dialysable calcium is presumably in combination with proteins [Marrack and Thacker, 1926], fatty acids and perhaps other compounds. By passing in an excess of CO_2, we should expect that the calcium would be split off from combination with these compounds and that it would appear as calcium bicarbonate in the solution. In the splitting of complement by this method, therefore, the globulin fraction should contain little or none of the calcium present in the serum. This we have found to be the case, for all the calcium invariably passes into the albumin fraction. Furthermore, if calcium oleate is added to heated rabbit serum as in Liebermann's preparation, and then subjected to CO_2 treatment all the added calcium is found in the albumin fraction. According to this view of the nature of complement, therefore, the splitting of complement by dilute acids can be explained on purely chemical lines, but in our opinion the problem is not quite so simple although this may be part of the true explanation.

If the active principle of complement is a calcium soap and if this substance or closely related compounds are solely responsible for the specific haemolytic properties, the protein and other serum colloids being concerned only indirectly, true splitting of complement should only be possible with acids which, like CO_2, give a soluble calcium salt. Thus if CO_2 separates the complement into two fractions, a globulin precipitate containing the insoluble higher fatty acids, and a "soluble" albumin fraction containing calcium bicarbonate, all acids which give soluble calcium salts should have a similar effect, whereas acids such as sulphuric and oxalic would precipitate the calcium in the wrong fraction. Only those acids giving soluble calcium salts should therefore give complete separation of complement into two separately inactive fractions identical with those obtained by CO_2 treatment. A large number of experiments have been carried out with various acids and the fractions obtained compared with the normal mid-piece and end-piece of the CO_2 separation. The chemical separation obtained agrees in every respect with the theory, for all or practically all the calcium was found in the albumin fraction when CO_2, hydrochloric or acetic acids were used and it was mainly in the globulin fraction in the case of oxalic acid. When sulphuric acid was used calcium was present in both fractions, mainly in the albumin fraction, a result which was expected since calcium sulphate is somewhat soluble. With

oxalic and sulphuric acids it was found that the best results were obtained when the acids were allowed to act for some considerable time (2 hours) and this was attributed to the formation of insoluble calcium salts on the non-ionised complex calcium compounds and the consequent time required for the removal of calcium from these compounds. The results with reference to complement activity showed a general support of the calcium soap view but suggested that, while this theory is partially correct, there are other factors to consider. The fractions obtained with acids giving soluble calcium salts appeared identical in all respects with those obtained with CO_2, but in the case of sulphuric and oxalic acids, the albumin fractions, which contained less calcium, and that probably in a different form, had little or no completing action for fresh CO_2-globulin fraction. In all cases the globulin fractions were able to activate the CO_2-albumin fraction. The only differences appear therefore in the albumin fractions, those from acids giving soluble calcium salts having the power to complete CO_2 mid-piece, whilst those from acids giving insoluble calcium salts have no such power. The re-formation of the complement by addition of each end-piece to its respective mid-piece fraction also gave some support to this theory, for full complement power was regenerated in the case of hydrochloric, acetic and similar acids, but not in the case of sulphuric or oxalic acids. The results were not as definite, however, as would be the case if this calcium soap view covered the whole explanation and there are probably other factors concerned. We suggest, therefore, very tentatively, that if calcium or some other salt of a fatty acid is the active haemolysin, the whole of this substance may not be present as such in the original serum at the beginning of the process of haemolysis. If this is so, separation of the calcium soap present will take place in the manner indicated according to the nature of the acid used, but this does not take into consideration the presence of other substances which may be concerned and which may be responsible for the renewal of the supply of the active agent.

Liebermann's views on the nature of complement have also been investigated in the light of the facts known about the non-dialysable calcium in serum. According to the general view, this non-diffusible calcium is chiefly in combination with protein and the higher fatty acids, and there is probably some state of equilibrium between the diffusible and the non-diffusible calcium. Loeb and Steinberger [1924], for example, found that only part of the calcium would diffuse out into water, the amount being sometimes as low as 55 % of the total calcium, but when dialysed against 0·8 % NaCl at p_H 7·4 or HCl at p_H 2·5, all the calcium was diffusible. This complete removal of calcium by dialysis against physiological saline is not supported by other results and our own experiments do not agree with this view. As a result of dialysis experiments of varied nature we have found that the removal of all the dialysable calcium does not inactivate complement. The diffusible calcium does not function therefore in the complement reaction except perhaps in the maintenance of a certain amount of calcium in combination with

proteins and fatty acids. If the calcium in serum is necessary for immune haemolysis it is only that which is present in the non-dialysable form, and according to the calcium oleate theory, any mechanism which tended to decrease the amount of non-dialysable calcium would decrease the haemolytic power. Ultra-violet light has a destructive action on complement [Courmont, Nogier and Dufourt, 1913], and according to Clark [1923] ultra-violet radiations increase the proportion of dialysable calcium in serum, although a conflicting result has been obtained by Moritz [1925]. A possible explanation of the destruction of complement by ultra-violet light, however, is that the physical conditions of the protein components are altered, and if any decrease in non-diffusible calcium does take place it could be explained by the decreased ionisation of the proteins or decreased affinity for calcium ions.

Interesting results have been obtained by dialysing the separated mid-piece and end-piece fractions against water and 0·9 % NaCl. In order to have calcium in both fractions, the saline used in the experiments was made up from the commercial product which contained a known small amount of calcium salts. The separate mid-piece and end-piece fractions therefore both contained calcium; the mid-piece by virtue of that in the sodium chloride added, whilst the end-piece contained all the calcium originally present in the serum together with that in the saline. The calcium in the globulin or mid-piece fraction was practically non-dialysable, whilst that in the albumin fraction was partly dialysable. These fractions were also dialysed against the calcium-containing 0·9 % NaCl. The globulin fraction gained calcium in the process of dialysis, the concentration of calcium inside the sac being greater than that outside at the end of the process. The globulin fraction thus has the power of combining with inorganic calcium to form non-diffusible calcium compounds. The albumin fraction appears to have enough calcium present to supply the demands of the proteins, etc., and it loses diffusible calcium when dialysed against water or 0·9 % NaCl. In the "regeneration" of complement by the addition of the albumin fraction to the globulin fraction, the latter, which contains no calcium, will take it up from the former and will regenerate non-diffusible calcium compounds. Thus these facts are in no way antagonistic to Liebermann's view.

Several considerations have led us to investigate the possibility of calcium being the fourth component or relatively heat-stable factor inactivated by ammonia (see preceding paper), the main factor being that the splitting of complement by CO_2 results in the separation of all the calcium in the albumin fraction in which the fourth component is found. In addition, if Liebermann's view is correct, the calcium from the active calcium oleate would be in that fraction. The mechanism of the process of inactivation by ammonia could possibly be explained along these lines by the formation of a non-ionised double salt of calcium and ammonium, and possibly phosphate, which would disturb the equilibrium between the dialysable and non-dialysable calcium and lead to a diminution of the amount of calcium combined with protein and fatty acids.

Repeated experiments have shown, however, that no increase in dialysable calcium occurs during the process of inactivation by ammonia and this view does not appear to be the true explanation of the inactivation process. On the other hand, there is a fair amount of evidence to suggest that calcium does function actively in complement action and is connected with the fourth component. Over an extended period calcium estimations have been carried out with the fractions of complement, and these have been compared with the power of such fractions to reactivate ammonia-inactivated serum. A general parallelism has been found, and in practically every case there is correlation between calcium content and the amount of the fourth component present. Thus calcium-free mid-piece fractions do not contain this component, whilst the end-piece fraction, which contains practically all the calcium originally present in the serum, is always rich in this factor. In addition the albumin fractions obtained when sulphuric and oxalic acids are used to split the complement contain little or none of this component and these fractions contain less calcium than the CO_2-albumin fraction and that present probably in an altered condition. Attempts have been made to reactivate ammonia-treated serum by the addition of small amounts of calcium salts or by the use of saline containing small amounts of calcium, but these have been unsuccessful, as have all our attempts to replace the fourth component by calcium. More evidence on the exact nature of the calcium compounds present in serum may be required before we can reproduce the same conditions artificially once the equilibrium has been disturbed.

Experimental.

The details for the separation of complement by CO_2 and for the inactivation by ammonia are described in the previous papers. The calcium estimations were carried out by Tisdall's modification [1923] of the method of Kramer and Tisdall [1921], and the amount of non-dialysable calcium was estimated after dialysis through collodion sacs [Moritz, 1925] or through parchment thimbles. Physiological saline made from pure NaCl (A.R.) was used for all purposes unless otherwise stated.

Dialysable calcium and complement action.

Guinea-pig serum was dialysed against water in collodion sacs under a negative pressure and against water and 0·9 % NaCl in parchment thimbles. The sera free from all dialysable calcium were still haemolytic towards sensitised cells and all contained calcium.

Similarly end-piece fraction dialysed alone against water or 0·9 % NaCl retained part of its calcium and also its full power to activate fresh mid-piece. This dialysed end-piece, even when heated at 56°, still contains the fourth component and will reactivate ammonia-treated serum.

Exp. Neutralised end-piece (CO_2) was dialysed in parchment thimbles for 24 hours against water and then made isotonic with NaCl. 60 % of the calcium dialysed out (average of several determinations) but the solution still retained the power to activate fresh mid-piece and also ammonia-inactivated serum.

1·0 cc. NH_3-inactivated serum	... .	–
0·5 cc. ,,	+0·5 cc. 0·9 % NaCl	–
0·5 cc. ,,	+0·5 cc. dialysed end-piece ...	+ + +
0·5 cc. ,,	+0·5 cc. heated dialysed end-piece	+ +
0·5 cc. fresh mid-piece	+0·5 cc. dialysed end-piece	+ + +

The separation of complement by CO_2 and the distribution of calcium.

Calcium estimations with the separate mid-piece and end-piece fractions (CO_2) showed that practically all the calcium in the serum passes into the albumin fraction. The same result was obtained with rabbit serum and if calcium oleate had been added previously, as in the preparation of Liebermann's artificial complement, this added calcium was always found in the albumin fraction.

Calcium estimations (mg. per 100 cc. of serum or equivalent amount of the fractions).

Table I. (*Guinea-pig serum.*)

In guinea-pig serum	10·5	8·5	7·8	—	—	8·0	11·0	8·0	—
In mid-piece fraction (CO_2)	0	0	0·4	0	0	0	0·3	0	0
In end-piece fraction (CO_2)	10·0	8·5	7·6	9·2	6·0	8·5	10·5	8·5	12·0

Table II. (*Rabbit serum.*)

Rabbit serum	12·8	6·0	12·0
Mid-piece (CO_2)	0	0	0
End-piece (CO_2)	13·2	5·8	11·8
Rabbit serum + calcium oleate (serum X)	20·0	12·8	16·8
Mid-piece of serum X	0	0	0
End-piece of serum X	20·4	12·3	17·0

Separation of complement by various acids, the calcium distribution and the fourth factor content.

Different acids were added to serum to give the same resulting p_H as that produced by passing CO_2 through for 15 minutes as in the ordinary method of separation. The sera were diluted to 1 in 10 with distilled water, allowed to stand for 1–2 hours, and centrifuged. The fractions were adjusted to p_H 7·5 and made isotonic with serum by means of pure NaCl. The results varied but in general the separation with acetic acid, phosphoric acid and HCl ran parallel with that by CO_2, while oxalic, sulphuric and, to a lesser extent, citric acids did not give the same results. Thus the separated fractions obtained with the latter series of acids were not active when added together and this we attributed to the differences in the distribution of the calcium. The following is an average result, the separate mid-piece and end-piece fractions being inactive in all cases even when used in double quantity.

Table III.

	CO_2	Acetic acid	HCl	H_2SO_4	Oxalic acid
0·5 cc. mid-piece + 0·5 cc. end-piece	+ + + +	+ + + +	+ + + +	+	–
0·5 cc. CO_2 mid-piece + 0·5 cc. different end-pieces	+ + + +	+ + + +	+ + + +	+ +	–
0·5 cc. CO_2 end-piece + 0·5 cc. different mid-pieces	+ + + +	+ + + +	+ + + +	+ + +	+ + +

In nearly every experiment the mid-piece fractions from the oxalic acid and sulphuric acid separations had a slight anticomplementary action when tested with small amounts of guinea-pig serum and this is presumably due to the insoluble calcium salt precipitated on the globulin. The anticomplementary power, however, is not responsible for the inactivity of the "re-formed" complement in these cases, for these mid-piece fractions can fully activate fresh CO_2 end-piece.

The separated fractions were then tested with ammonia-inactivated serum (see previous paper) and the calcium content of each fraction was also determined. In general the power to reactivate the ammonia-treated serum was only evident in solutions containing calcium. The converse was not always true but the inability of the globulin fractions with H_2SO_4 and oxalic acid to function in this way can be attributed to the fact that the calcium is present as an insoluble salt. The occasional slight power of the oxalic and sulphuric acid end-pieces to reactivate this inactive serum may be due to the calcium still left in the solution or to calcium from other sources, and in this connection it must be remembered that calcium is always present in the red cell [Parnas and Jasinski, 1922] and will be liberated as soon as any haemolysis occurs.

To obtain the best distribution of the calcium with the different acids it was found necessary to allow them to act upon the serum for 2 hours. The acids thus appear to split up the complex calcium compounds slowly, especially where the acid gives an insoluble calcium salt.

Table IV.

	CO_2		HCl		Oxalic acid		H_2SO_4	
	mid-piece	end-piece	mid-piece	end-piece	mid-piece	end-piece	mid-piece	end-piece
Calcium content	0	7·4	0	7·5	6·0	1·6	1·2	6·2
(mg. per 100 cc. of	0	8·4	—	—	6·2	2·4	2·4	6·0
the original serum)	0	7·6	—	—	6·6	0·6	0·4	7·6
	0	9·2	0	9·0	8·6	0·6	2·8	6·6
Fourth component	None	Present	None	Present	None	None or trace	None	Trace

The inactivation by ammonia and dialysable calcium.

Serum was inactivated by ammonia (see preceding paper) and the amount of dialysable calcium determined after dialysis in collodion sacs [Moritz, 1925] or in parchment thimbles for varying periods of time. Control experiments

in which serum was treated with one-quarter of its volume of 0·9 % NaCl and incubated for the same time showed that no increase in dialysable calcium had taken place during the inactivation process.

Table V. *Calcium estimations* (*mg. per* 100 *cc. of serum or equivalent amount of solutions*).

Guinea-pig serum	13·0	10·8	5·6	—	—
Non-dialysable calcium in NH_3-inactivated serum	7·0	3·0	3·1	5·8	3·9
Non-dialysable calcium in control	7·5	3·0	3·2	5·0	4·1

SUMMARY.

(1) The relationship between the calcium in serum and complement activity has been investigated. The dialysable calcium is not essential for complement action but may be concerned indirectly on account of the equilibrium between dialysable and non-dialysable calcium. The non-diffusible calcium appears to be connected in some way with complement activity.

(2) The calcium content of various fractions of serum obtained by using CO_2 and other acids and the power to reactivate ammonia-inactivated serum show some correlation. Attempts to reactivate ammonia-treated serum by the addition of calcium in various forms were however unsuccessful.

(3) When complement is split by CO_2 all the calcium passes into the albumin fraction. If calcium oleate is added all the added calcium is found in the albumin fraction. The possibility of the chemical separation of complement by dilute acids, if complement consists of calcium soaps as in Liebermann's theory, is discussed.

(4) Acids which give insoluble calcium salts, such as oxalic and sulphuric, distribute the calcium differently, calcium being found in both fractions, in the case of oxalic acid mainly in the globulin fraction. The fractions obtained when these acids are used are inactive themselves and the combined fractions have little or no activity. Hydrochloric, acetic and phosphoric acids behave like CO_2.

(5) No alteration in dialysable calcium occurs during the inactivation by ammonia.

Two of the authors (A. W. and J. G.) beg to express their indebtedness to the Medical Research Council for a grant in aid.

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CXXXI. THE FOURTH COMPONENT OF COMPLEMENT AND ITS RELATION TO OPSONIN.

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(*Received August 21st, 1926.*)

The question of the nature of opsonins in normal serum has engaged the attention of many workers during the last twenty years. Broadly speaking, there appear to be two schools of thought. Many workers, including Neufeld and Hühne [1907] and Levaditi and Inmann [1907], consider that opsonin is intimately related to, if not identical with, the haemolytic complement in serum, whilst others, including Wright and Douglas [1904], believe that opsonins are distinct bodies not identical with complement or anti-bodies. Briefly, the evidence in favour of the first view is that processes that absorb complement from serum generally remove the opsonic power. Thus any system such as bacteria, serum or red cells plus the immune body will take up complement from a normal serum, and the serum at the same time loses its opsonic action. Browning [1925], however, shows that bacteria may remove most of the opsonins from serum and still leave a considerable amount of haemolytic complement. This he explains either by the existence of a natural immune body which combines with the organism and leads to the fixation of a definite amount of complement or to the existence of multiple complements, some of which only are concerned with opsonic action. The effect of heat upon the opsonic and complementary properties of serum seems in favour of the identity of the two properties; both are definitely destroyed at 56°.

It seemed to us that some advance would be made if the question of the identity or non-identity of complement and opsonin could be settled. In the first place it must be pointed out that even if some process, which removes complement activity from serum, removes opsonic activity at the same time, it is not necessarily proved that the two substances are one and the same.

For since complement involves several factors, all of which must be present to bring about haemolysis, opsonin may be similarly complex and we may destroy one factor which happens to be common to both. Thus the evidence mentioned above must be taken to indicate that one factor of complement at least is also concerned in opsonic action; other complement

factors may not be concerned. If complement could be inactivated by destroying one factor and the opsonic power of the serum remained unimpaired then the non-identity of the two properties or substances would be established. In a foregoing paper we have described a simple and easily applied method for destroying or inactivating a relatively heat-stable component of complement and it was considered of interest to determine whether this factor had any connection with opsonic action.

Serum was treated with one-quarter of its volume of $N/6{\cdot}5$ NH_4OH and incubated for various times ranging from half-an-hour to 2 hours. It was neutralised with acid and then tested for complementary and opsonic power against a control which had been incubated for the same time and diluted to the same extent with normal saline. The opsonic tests were carried out by mixing equal volumes of serum, suspension of leucocytes and suspension of organisms, and incubating the mixture for half-an-hour at 37°. Slides were stained and the presence or absence of phagocytosis was determined. The experiments were performed with rabbit and guinea-pig serum, the respective leucocytes and with staphylococci and tubercle bacilli. Controls with washed leucocytes and bacteria alone showed practically no phagocytosis.

The treatment with ammonia described above never failed to give a serum with no complement activity, due simply to lack of the fourth component, and on the other hand such serum proved to have lost none of its opsonin. In no experiment could it be distinguished from the control of unheated serum so far as phagocytosis was concerned.

Thus it is definitely established that while complement and opsonin may be closely related the whole complement system is not essential for opsonic action.

SUMMARY.

Serum in which the fourth component of complement had been inactivated by means of ammonia proved to have lost none of its opsonic activity. The fourth component of complement therefore plays no part in opsonic action.

The authors beg to express their gratitude to the Medical Research Council for a grant in aid to two of them (J. G. and A. W.) and to Professors H. J. Hutchens and J. W. McLeod for facilities granted.

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CXXXII. THE EFFECT OF FAT ON THE TRYPTIC DIGESTION OF PROTEIN *IN VITRO*[1].

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That fat retards the digestion of protein in the body is an established fact (Luciani [1913], Pawlow and Thompson [1910], Cowie and Mumson [1908], Carlson [1914, 1915], Lockwood and Chamberlin [1923], Frank [1923]), but an explanation of this action has not yet been given.

It has been the aim of the following work to investigate one part only of the chemical aspect of the subject and not to reproduce the conditions of digestion in the alimentary canal. Further, protein was digested *in vitro* in the presence of a proteolytic enzyme free from any lipase, and thus no digestion products of fat were involved.

Method.

A solution of egg-albumin, p_H 1·7, was digested with pepsin for 24 hours at 37°, as such treatment accelerated the subsequent digestion with trypsin. Tryptic digestion was carried out in $M/10$ buffer solution of sodium phosphates, starting at p_H 8·0, in the presence of an emulsion of olive oil and controlled by a similar experiment in which the oil was omitted. The rate of tryptic digestion was determined by Sørensen's method.

Peptic digestion.

A solution was prepared containing

15 % egg-albumin (dried).
3 % pepsin (pepsin B.P. Allen and Hanbury's).

This was brought to p_H 1·7 by means of hydrochloric acid heated to 37° and was incubated at this temperature for 24 hours. Sodium hydroxide was added until the p_H reached the isoelectric point of denatured albumin [Chick and Martin, 1911, 1912].

It was heated for 30 minutes over a bath of boiling water; cooled for 15 minutes by means of running water and filtered through a Buchner funnel. This solution of the substrate was kept saturated with toluene and at a p_H of 5·5. The amounts of total nitrogen and, in the later work, the amino-acid

[1] An abridgment of a thesis for which the degree of Master of Science of the University of London was awarded.

nitrogen present in the solution of the substrate were estimated by the Kjeldahl method and Sørensen's method respectively, and from these results the amount of nitrogen available for digestion was determined.

Tryptic digestion and oil experiments.

Tryptic digestion was carried out in $M/10$ buffer solution of sodium phosphates. Liquor Trypsin Co. (Allen and Hanbury's) was used and olive oil as fat. The volume of $N/10$ NaOH was determined, for each sample of olive oil, which was required to bring 100 g. of oil in 100 cc. of absolute alcohol to that definite pink colour in the presence of phenolphthalein which was used as an end-point in later titrations.

Emulsion of oil. 1 part by weight of finely powdered gum acacia and 4 parts olive oil were mixed thoroughly in a mortar and then 2 parts of water were added, and the mixture stirred briskly until a permanent emulsion was obtained. The emulsion could be diluted with the substrate solution without separation of oil. Trypsin was added to the buffered solution of substrate until the resulting liquid contained 10 % of the former, and then NaOH until the p_H of the liquid was 8·0.

To equal volumes of buffered solution of substrate and trypsin (p_H 8·0) were added, in the emulsion experiment, the required weight of emulsion and in that of the control a weight of gum equal to that which was present in the emulsion, dissolved in such a volume of water as to bring this solution of gum to the same volume as that of the emulsion. In each case the liquids were saturated with toluene.

Two alternative methods were then employed.

A. The control mixture was brought to p_H 8·0 by the addition of NaOH solution and an equal volume of NaOH solution was added to the emulsion mixture.

B. Both control mixture and emulsion mixture were brought to p_H 8·0.

The capillator method of determining p_H was used.

The emulsion and control mixtures were brought in equal times and with constant stirring to 37° and incubated at that temperature in a water-bath for as long as the experiment lasted.

The amount of digestion was determined by Sørensen's method of direct titration in the presence of formaldehyde. A sample was taken before digestion began both from the emulsion mixture and the control and then from each at subsequent intervals during the experiment. These samples were titrated with $N/10$ NaOH until a definite pink colour was produced in the presence of phenolphthalein (p_H 8·8), and this volume, less that required before digestion, was taken as representing the degree of digestion which had occurred. It was decided that production of acidity as well as of amino-acids could be taken as an indication of the degree of digestion [cf. Lannoy, 1918].

The maximum error of this method was shown to be 0·52 cc. on a reading of 20 cc. whilst the average error was 0·08 cc. on that volume.

The following curves show the results of typical experiments:

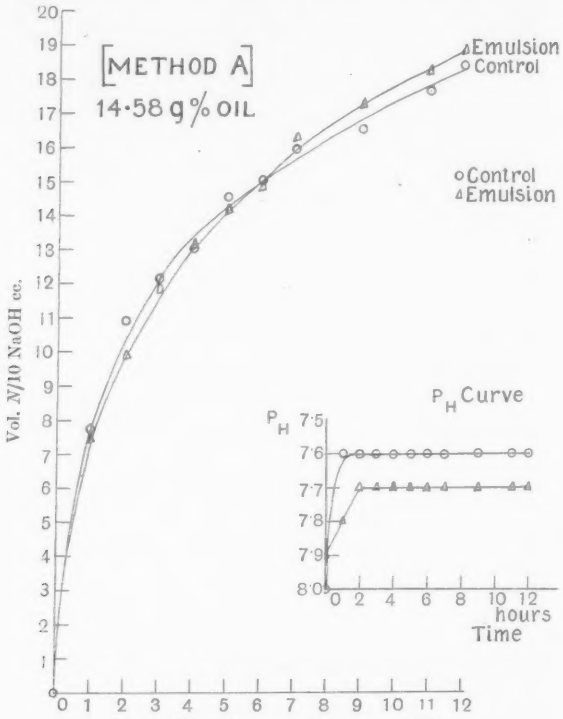

Fig. 1. Digestion curves (experiment 33).

Summary of Results.

Method A. In those cases in which the control mixture was brought to p_H 8·0 by the addition of alkali and an equal volume of alkali was added to the emulsion mixture, no definite proof that oil in the form of an emulsion has any effect on the digestion *in vitro* was obtained, as the variations which occurred were almost all within the experimental error.

It is interesting to note, however, that the lower percentages of oil were insufficient to alter the p_H of the emulsion mixture perceptibly (see Fig. 2), but in the presence of more than 13 % of oil the p_H of the emulsion mixture was 7·9 before digestion began, whilst that of the control was 8·0 (see Fig. 1) and that it became higher than the control after 1 hour's digestion and remained so throughout. Even so, as the result of 39 experiments, no definite increase in digestion was evident.

In every one of these experiments, however, a small initial retardation in the presence of oil was noted followed by a later acceleration (see Figs. 1

and 2). Though such variations from the control were practically all within the experimental error, the phenomenon was so regularly obtained that it is felt that the work would be worth repeating with a still more sensitive method of p_H estimation.

Method B. On the other hand, if the emulsion mixture as well as the control were brought to p_H 8·0 the emulsion experiments showed an increased amount of digestion (6 experiments). The p_H of the emulsion mixture became higher than that of the control from the first hour and remained so throughout the experiment (see Fig. 3).

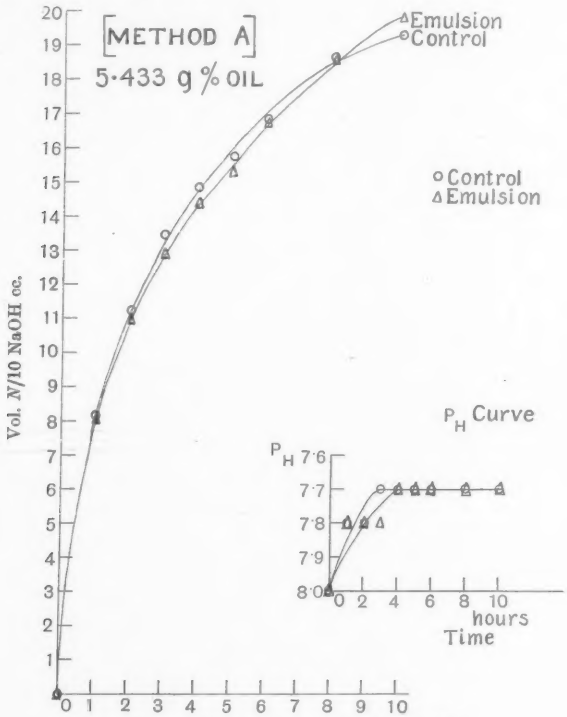

Fig. 2. Digestion curves (experiment 38).

Discussion of Results.

In connection with the foregoing results certain factors may be involved.

(1) In those experiments in which the control and emulsion mixtures were brought to p_H 8·0 before digestion, oil in the form of an emulsion exerted a small accelerating effect on tryptic digestion. This acceleration increased as digestion proceeded, but never amounted to more than 10 %. This is probably connected with the fact that the p_H of the emulsion mixture never

fell as low as that of the control mixture. Sørensen has shown that trypsin is very sensitive to any change in hydrogen ion concentration.

Further, oil in the form of an emulsion may exert some action on the substrate. It was noticed that on the addition of alkali to the control mixture a flocculent precipitate appeared, but that in samples of the emulsion mixture which had been allowed to stand only a fine precipitate was evident. This fact, according to the observations made by Fodor [1922], would account for an increase in digestion in the emulsion mixture.

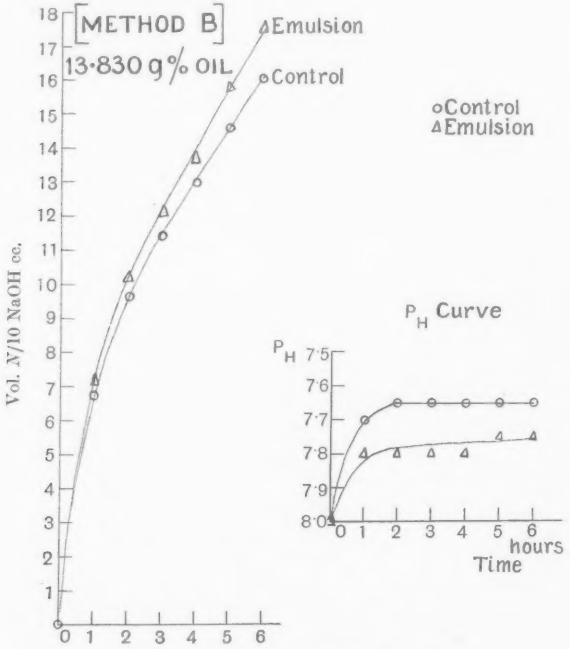

Fig. 3. Digestion curves (experiment 45).

Oil in the form of an emulsion may exert some protective action on trypsin itself. Northrop [1922] has shown that the normal action of trypsin is retarded by an inhibitor produced in the course of digestion. It is possible that oil may protect trypsin from the action of this substance, and also from the action of alkali.

(2) In those cases, however, in which the control mixture was brought to p_H 8·0 and an equal volume of alkali added to the emulsion mixture no definite effect of olive oil was observed, but a higher p_H was recorded in such concentrations of oil in which variations in p_H could be appreciated. As trypsin is very sensitive to any change in hydrogen ion concentration, it follows that

fat probably exerts some retarding action to balance the higher p_H of the emulsion mixture. Such a retarding action would most likely have entered into the experiments discussed under heading (1), but in these cases the retarding action was not sufficient to equalise the digestion in emulsion mixture and control.

It has been shown that surface plays a very important part in enzyme action. Oil reduces surface tension and so lowers surface energy, hence a retardation in digestion would be expected. Meyerhof [1914] has shown that the inhibiting effect of alcohols on enzyme action varies as their power to lower surface tension.

Bayliss [1911] obtained a similar result. He found that saponin reduced surface energy and had thus a retarding action on the effect of urease on urea. He [1906] also demonstrated the fact that adsorption takes place between a substrate and enzyme. It is possible that the presence of fat may affect the rate of formation of this adsorption compound.

At the present stage it is impossible to say how far each of these factors is concerned, but the gross results of the foregoing experiments show that olive oil, unhydrolysed and in the form of an emulsion, does not retard the action of trypsin *in vitro* on albumin previously digested with pepsin. The explanation of the retarding action of fat on the digestion of protein *in vivo* may be found either in the action of the products of the hydrolysis of fat or in some specific action of fat in the alimentary canal.

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CXXXIII. FAT CONTENT OF BREADS AND CEREALS.

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(*Received August 24th, 1926.*)

Up to the present there has been some doubt as to the absolute fat content of the different cereals and breads. Smedley MacLean [1922, 1923] states that by first hydrolysing yeast by boiling with *N* HCl for 2 hours, filtering, drying the residue and subjecting it to ether extraction in a Soxhlet apparatus, she obtained much larger quantities of fat than by direct extraction of the dried yeast with ether. Sharpe (unpublished results), working in this Institute, found that in the case of breads much larger quantities of fat could be obtained by first digesting the bread.

His results were so striking that it was felt that further investigation should be made.

It was decided to extend the study to the flours prepared from the chief cereals and from soya bean.

A. *Breads.*

The breads were dried, finely ground in a mortar, dried to a constant weight and a weighed sample extracted with ether in a Soxhlet apparatus for 9–12 hours.

Another sample was digested with 0·2 % HCl and 0·05 % pepsin, 25 cc. being used; the digest was repeatedly extracted with ether in a separation funnel, the ether evaporated off and the residue dried and weighed.

Results.

	Fat	
	Soxhlet %	Ether extraction after preliminary digestion %
Scotch white wheaten	0·29	0·93
English white wheaten	0·27	0·61
Scotch pan loaf	0·96	1·6
Scotch brown bread	1·4	2·8

In the Scotch white wheaten bread, Soxhlet's extraction gave 0·29 %, whilst the digestion method gave 0·93 %. Plimmer [1921] attributed the higher fat content in Scotch bread to the practice of adding milk to the dough. This is not the case.

B. *Cereal flours and soya bean flour.*

The meals were finely ground in a mortar and all were passed through a fine sieve so that they were in the same state of subdivision. The powder was then dried to constant weight.

A weighed sample was extracted by Soxhlet's method and another digested with hydrochloric acid and pepsin, and the digest extracted in the same way as in the case of breads. Three analyses were made in every case.

The results (I and IV) show that, when cereal flours were treated in these two ways, the marked differences observed in breads did not occur, and that digestion does not greatly increase the yield of fats.

It seemed desirable to ascertain (i) whether digestion after Soxhlet's extraction gives a larger yield of fats, (ii) whether more complete disintegration of the flour by further trituration in the course of extraction allows of more complete extraction of fats, and (iii) whether preliminary digestion of the flour increases the yield of fats by Soxhlet's method.

Methods.

(i) The meal was extracted by Soxhlet's method with ether and the fat weighed. The meal residue was treated with HCl and pepsin for 24 hours, dried, pulverised and extracted in a Soxhlet with ether and the extract dried and weighed.

(ii) A weighed portion was extracted in a Soxhlet apparatus with ether and the fat weighed. The residue was then pulverised and again extracted in a Soxhlet apparatus with ether, the extract being again weighed. This was repeated until negligible quantities of fat were obtained. Finally, the meal residue was treated with HCl and pepsin for 24 hours at 38°, the digest dried and extracted in a Soxhlet apparatus with ether and the extracted fat weighed.

(iii) A weighed portion of the meal was digested with HCl and pepsin, the digest dried, pulverised and extracted with ether in a Soxhlet apparatus and the fat weighed.

Results. (I) Extraction and subsequent digestion:

	Fat	
	Soxhlet extraction %	Extraction in Soxhlet after subsequent digestion %
Wheat	1·65	1·99
Oats	7·13	7·62
Barley	1·94	2·38
Soya bean	17·15	18·24

This shows an increased yield of about 6 % after digestion.

(II) Extraction and repeated extraction with trituration, then subsequent digestion:

	Fat		
	First extraction %	After four triturations %	Total %
Wheat	1·65	2·14	2·16
Oats	7·14	7·70	8·03
Barley	1·95	2·68	2·73
Soya bean	17·21	18·65	19·22

(III) Extraction after digestion and drying:

	Fat %		Fat %
Wheat	1·65	Barley	1·92
Oats	7·15	Soya bean	16·7

(IV) Extraction in separator after digestion:

	Fat %		Fat %
Wheat	1·71	Barley	1·94
Oats	7·16	Soya bean	17·05

The analyses of barley show a wide discrepancy and Plimmer's results diverge widely from other analyses.

Summary.

1. Soxhlet's extraction with ether removes only a small proportion of the fats from breads, whilst extraction after peptic digestion removes two or three times as much.

2. The usually accepted average of 0·3 % of fats in dried wheaten bread, which seems disproportionately small when compared with the 2 % in wheaten flour, is altogether too low. In white bread it is more nearly 0·6–0·9 %.

3. In flours, Soxhlet's method, as usually carried out, does not extract all the fats. A further amount of about 6 % may be separated after digestion.

4. Soxhlet's method with repeated trituration of the flour in the course of extraction secures a very complete removal of the fats and gives a higher result than extraction by Soxhlet after digestion or by extraction in a separator after peptic digestion.

5. The remarkable difference between the proportion of fat extracted by Soxhlet's method from bread and from flours must be due to some change produced in the process of baking

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CXXXIV. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. IV.

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(*Received August 24th, 1926.*)

It was shown in the preceding communications of this series [Zilva, 1924, 1, 2; 1925] that concentrated antiscorbutic fractions could be obtained from decitrated lemon juice by removing from it sugar and substances precipitable by alcohol and by neutral lead acetate and eventually precipitating from the filtrate the active principle with basic lead acetate. Such an active fraction contains only about $\frac{1}{500}$th of the dry matter of the original lemon juice but is undoubtedly a mixture. It was thought that it would be instructive at this stage of the inquiry to gain information about the behaviour of some of the inorganic constituents of lemon juice in the process of fractionation. Attention was mainly directed to the original juice, to the decitrated juice and to the purest fraction, *i.e.* the basic lead acetate fraction obtained after removal of the citric acid and impurities precipitable by alcohol and by neutral lead acetate. The sugar was not removed by fermentation previous to precipitation and the small amount which was brought down in this fraction by the basic lead acetate was ignored, as its removal by fermentation would most probably have been attended by the introduction of some of the inorganic constituents of the yeast.

The purest fraction was obtained by treating the decitrated lemon juice with neutral lead acetate, and centrifuging. Without removing the excess of neutral lead acetate, the supernatant fluid was then precipitated with basic lead acetate and centrifuged again. The precipitate which contained the active principle was dissolved in acetic acid, decomposed with magnesium sulphate, treated with two to three volumes of absolute alcohol, filtered and evaporated *in vacuo* to a small volume in order to remove the alcohol. In a few cases, mostly in the early experiments of this investigation, the basic lead acetate precipitate was decomposed with hydrogen sulphide. This method of removing the lead was discarded since it is uncertain whether the antiscorbutic factor withstands such treatment. As was already pointed out by one of us [Zilva, 1924, 2], test guinea-pigs do not thrive on fractions previously decomposed with hydrogen sulphide owing, most probably, to the toxic action of traces of mercaptan formed during the decomposition. On the other hand, the removal of the lead

by magnesium sulphate was found to be suitable for the biological tests of the antiscorbutic factor and it was, therefore, desirable to follow the technique which proved to yield active preparations.

Total ash.

As one would expect, the inorganic contents of lemon juice and the different elements constituting the ash vary within certain limits. Thus Oliveri and Gerrieri [1895] found *Citrus Limonum* L. to contain 0·20 % of ash, whilst Colby and Dyer [1890, 1891–2, 1893] found the juice from the Californian lemon to contain 0·63 %, 0·54 % and 0·78 % of inorganic constituents.

The following table gives five representative analyses of the ash of lemon juice as quoted by König:

Table I.

No.	Fe_2O_3—Al_2O_3 %	CaO %	MgO %	K_2O %	Na_2O %	P_2O_5 %	SO_3 %	SiO_2 %	Cl %
1	1·32 3·28	15·79	5·10	58·18	—	10·89	2·63	2·31	0·50
2	0·28	34·07	4·00	45·33	2·03	10·19	2·74	0·54	0·51
3	0·58	25·67	4·80	61·20	1·50	12·0	2·94	0·68	0·27
4	1·36	36·07	5·58	30·05	3·0	19·63	2·48	0·85	0·27
5	(2·57)	8·71	4·45	46·94	2·53	5·32	2·00	0·91	0·96

NOTES. In no. 5 the Fe—Al content is given as the double phosphate. In nos. 2, 3 and 4 the Mn_3O_4 contents are 0·28, 0·28 and 0·71 % respectively.

We determined the total ash of lemon juice by straining the freshly expressed juice through muslin, evaporating 100 cc. of the liquid and incinerating. Six batches examined yielded the following figures:

0·1503 %, 0·2124 %, 0·1732 %, 0·2034 %, 0·1896 %, 0·2084 %.

The ash of the purest fraction obtained from each of the above juices was also estimated and found to be:

0·00262 %, 0·00069 %, 0·00252 %, 0·00790 %, 0·00601 % and 0·002742 %.

These figures are no doubt higher than they should have been since the ash contained traces of the inorganic reagents used in the fractionation, such as calcium from the calcium carbonate employed in the decitration of the lemon juice. The lead acetate precipitates were in this case decomposed with hydrogen sulphide in order to obviate the introduction of minute quantities of magnesium. The figures, however, give us an idea of the order of the inorganic contents of the purest fraction.

The ash was examined for nickel, cobalt, boron, iodine, phosphorus, of which the first three were found to be absent from the purest fraction in quantities within the limits of the tests employed.

Nickel.

Tchugaeff's [1905] test was employed in detecting this element. A litre of lemon juice was fractionated as previously described and incinerated. The ash was taken up in dilute nitric acid and concentrated to 3 cc. Sufficient ammonium hydroxide was added to make the solution alkaline, after which

it was treated with 1 cc. of 1 % dimethylglyoxime solution, cooled and shaken with ether. The ethereal extract was colourless, nor was there a coloured zone between the aqueous and ethereal phases. Similar negative results were obtained with such preparations on two other occasions.

This test is claimed to be sensitive to 1 part in 400,000, yielding a pink-coloured nickel complex which rises, on shaking with ether, in a layer between the two liquids. On applying the test to a solution containing about $\frac{1}{30}$th mg. nickel as nickel sulphate in presence of cobalt nitrate and copper sulphate of similar concentrations, a very marked positive result was obtained.

Cobalt.

The purest fractions of three different litres of lemon juice were tested for cobalt, utilising Vogel's test [1879] for this element. The incinerated material was extracted with dilute nitric acid and made up to 10 cc. To this were added 15 cc. of concentrated ammonium acetate solution, 1 cc. of 5 % acetic acid and 5 cc. of saturated ammonium thiocyanate. This mixture was shaken with a little acetone, and a mixture of alcohol and ether (1 : 10). No coloration was produced.

In the presence of cobalt a blue coloration is formed in the ethereal layer. A control solution containing $\frac{1}{30}$th mg. of cobalt as cobalt nitrate in the presence of small quantities of copper sulphate and nickel sulphate, and ferric chloride gave a marked positive reaction.

Boron.

A preparation of the purest fraction equivalent to a litre of the original juice was evaporated to dryness at 105°. The residue was fused in a platinum crucible with four times its weight of sodium carbonate and the melt extracted with hot water and filtered. The filtrate was concentrated on the water-bath, acidified with hydrochloric acid and evaporated to dryness in the presence of a few drops of an alcoholic extract of turmeric. No coloration resulted. Only one batch equivalent to a litre of lemon juice was tested for boron. The test is claimed to be sensitive to $\frac{1}{500}$th mg. A blank containing $\frac{1}{50}$th mg. gave a positive reaction.

Iodine.

The method employed was Kelly and Husband's modification [1924] of Kendall's method. By this method the substance is fused with sodium hydroxide and the organic matter destroyed by potassium nitrate. The iodine which is fixed as sodium iodide is oxidised by bromine to iodic acid, liberated with potassium iodide and titrated with $N/200$ thiosulphate, using starch as the indicator.

The estimations were carried out on batches of the purest fraction equivalent to 1 litre of the lemon juice. The preparations were evaporated to dryness, and the residues treated according to the directions given by Kelly and Husband.

Four batches of the purest fraction equivalent to 1 litre of the original juice were examined separately, yielding 0·206 mg., 0·118 mg., 0·147 mg. and 0·118 mg. of iodine per batch. As the absolute limit of accuracy of the method employed is 0·1 mg. it may be concluded that the iodine content is 0·1–0·2 mg. per litre.

A blank experiment gave no coloration with starch.

It was previously shown [Zilva and Miura, 1921; Connell and Zilva, 1924] that the antiscorbutic factor did not dialyse to any appreciable extent in 3 days through collodion thimbles soaked in 88 % alcohol for 24 hours, whilst it did so through thimbles soaked in 95 % alcohol. Experiments were instituted to study the diffusibility of the iodine in antiscorbutic fractions as compared with that of simple inorganic iodides. Decitrated lemon juice was used as the source for the antiscorbutic vitamin and was dialysed in batches of 250 cc. contained in five thimbles of about 60–70 cc. capacity. The thimbles were closed with rubber stoppers and suspended by means of glass hooks fitted in the rubber. The dialysis, which lasted 72 hours, was carried out in distilled water which was changed every $2\frac{1}{2}$ hours during the day but not at night. At the end of this period the thimbles were emptied and well washed. The contents were then concentrated, incinerated and the iodine estimated as above. As will be seen from Table II, the iodine can have no relation to the active principle. It did not diffuse even through a 95 % thimble which permits the free passage of the antiscorbutic factor.

Methylene blue, Bismarck brown and neutral red diffused through the thimbles soaked in 95 % alcohol but not through those soaked in 88 % alcohol.

Table II.

Thimble index	Potassium iodide added (mg.)	Iodine (mg.)
Not dialysed	—	0·1180
88 %	—	0·1180
88	4*	3·14
95	—	0·1475
95	4	0·1770

* *i.e.* 3·06 mg. iodine.

Phosphorus.

This element was determined by Neumann's method. It will be seen from Table III that the phosphorus content of the purest fraction is of the same order as that previously observed [Zilva, 1924, 2].

Table III. *Percentage of* ***P.***

Raw juice	Basic lead fraction	Neutral lead fraction
0·00850	0·000517	0·000104
0·00700	0·000475	0·000115
0·00892	0·000554	0·000141
0·00806	0·000905	0·000110
0·00900	—	—

The various fractions were also examined for manganese and iron but some difficulties arose in connection with these tests and the matter is being investigated further.

Conclusions.

It is difficult to tell to what extent the figures obtained in this investigation are representative of lemon juice in general. One would naturally expect a certain amount of variation in the absolute values from juices of various origin and seasons. It is, however, doubtful whether the relative figures for the various constituents in the process of fractionation would be affected to any extent by these factors.

In the case of the elements not detected, it may safely be said that, unless it be in quantities which fall outside the limits of detection by the methods employed, they can have no bearing on the antiscorbutic activity of the juice.

Iodine may also be excluded in this sense since none of it diffuses through a collodion membrane soaked in 95 % alcohol, which indicates that it is present in a molecule much larger than that of the active principle. No appreciable quantities of the latter can be demonstrated in active solutions dialysed under the same conditions. This presence of iodine in a colloidal or semi-colloidal condition in lemon juice is, however, of interest.

Summary.

1. The behaviour of the inorganic constituents of lemon juice in the various stages of the preparation of concentrated antiscorbutic fractions has been studied.

2. Nickel, cobalt and boron were found to be absent; phosphorus and iodine were found to be present in the purest fractions.

3. The iodine present in the purest fractions is present in a compound of a colloidal or semi-colloidal nature, and is separable from the active principle by diffusion.

The authors wish to express their indebtedness to the Medical Research Council for whole-time grants.

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CXXXV. THE BIOLOGICAL SIGNIFICANCE OF *CIS-TRANS* ISOMERISM.

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(*Received July 22nd, 1926.*)

Researches on the relations existing between stereochemical structure and physiological action appear hitherto to have been promulgated almost entirely from one aspect of stereochemistry, that which is dependent on the presence of an asymmetric carbon atom, and invariably associated with optical activity.

When we come to the important class of compounds known as *cis-* and *trans-*, or geometrical isomers, we find an almost unexplored field of research. It was with a view to developing this branch of the subject, and at the same time throwing some further light on the fundamental problems of

(*a*) the relationship between chemical constitution and physiological and bactericidal action,

(*b*) the mechanism of the action of poisonous substances upon the more important constituents of protoplasm,

that the following researches were undertaken.

Hitherto the only work on this subject of which record can be found are some experiments by Ishizuka and others by Kahlenburg and True upon the relative toxicity of maleic and fumaric acids. Ishizuka [1897], experimenting upon dogs, found maleic acid to be much the stronger poison. Kahlenburg and True [1896], using *Lupinus albus* L., found the opposite effect, fumaric acid being the more toxic. On fungi, however, maleic acid again has a stronger action, *Penicillium glaucum* growing far more readily in solutions of fumaric acid than in those of maleic acid. This was confirmed by the present authors, who found that solutions of albumin decomposed rapidly in presence of fumaric acid owing to the growth of moulds, while maleic acid preserved them from putrefaction for a much longer period.

The chief compounds used in the investigation were maleic and fumaric acids, these substances being generally taken as typical of the ethylenic compounds which exhibit geometrical isomerism. Work was commenced upon the germicidal action of the foregoing compounds and a few similarly related compounds, and extended to their precipitating action on various proteins, their action on enzymes, and influence on the imbibition of water by gelatin, and also their distribution between water and proteins.

Germicidal action.

Some unexpected results had been obtained by Cooper, Woodhouse and Forstner [1925] in the course of their studies on the bactericidal action of maleic and fumaric acids. Difficulty had been experienced in obtaining consistent results, and as the organic matter in the nutrient medium had been suspected of causing the irregularities, a culture diluted 100 times was used. With the normal culture, using the Chick-Martin method at 20°, fumaric acid, the *trans*-isomer, appeared the stronger germicide; with the diluted culture the reverse held, and maleic acid became the more active. Carrying on the experiment at 37° a reversal again took place, fumaric acid being the stronger germicide.

In the bacteriological researches embodied in this thesis where the Chick-Martin method is being used, cultures which had been freed from organic matter either by dilution or by centrifuging were usually employed, normal cultures being used only for preliminary experiments.

The first experiments were carried out with maleic and fumaric acid, using normal cultures and cultures diluted 50 and 100 times respectively. These were mainly a check upon the earlier work and confirmed the conclusion that dilution of culture caused increase in germicidal power.

The results showed:

	Germicidal concentrations		
	Normal culture	Diluted culture	*B. coli communis*
Maleic acid	1 in 750	1 in 2500	37°
Fumaric acid	1 in 1000	1 in 3500	30 minutes' disinfection

The point of interest in these figures upon which most attention was concentrated was that although maleic, the *cis*-acid, was the stronger acid of the two, fumaric, the *trans*-isomer, was the more powerful germicide at 37°.

The object of diluting the cultures used has already been explained. The corresponding diminution in number of the organisms employed is an objection to the use of this method, however, and experiments were attempted with a centrifuged culture. A 24-hour culture of *B. coli* was grown in a sterilised centrifuge tube in ordinary lemco-peptone broth. This was then centrifuged for about half an hour and washed once with sterile Ringer's solution. The clear supernatant liquid was then replaced by Ringer's solution, the bacteria were well distributed and the culture thus obtained was used for the experiment. This method was found to give better results than the dilution method. The following result was obtained from a centrifuge experiment at 37°.

	Germicidal concentration
Maleic acid	1 in 1500
Fumaric acid	1 in 2500

To obtain further results from *cis*- and *trans*-compounds of this series, the methyl homologues, citraconic and mesaconic acids, were used. The same effect as with maleic and fumaric acids was observed, the *trans*-acid persistently showing higher germicidal power than the *cis*-acid.

The superiority of the *trans*-acids as germicides is of great interest when it is considered that the *cis*-acids are by far the stronger acids. Evidently bactericidal power is not determined alone by hydrogen ion concentration, but is associated with a selective action which is caused by configurational differences.

An attempt was next made to determine the inhibitory power of the acids. Maleic and fumaric acids caused so much precipitation of the peptone in the culture medium that the experiments were unsatisfactory.

The inhibitory powers of citraconic and mesaconic acids, which did not give excessive turbidity, could, however, be determined. The average results were as follows:

	Inhibitory concentration
Citraconic acid	1 in 500–750
Mesaconic acid	1 in 1000
Itaconic acid	1 in 500–750
Phenol	1 in 550

All three acids are seen to be more effective as germicides than phenol itself, and the *trans*-acid again proved to be more active than the *cis*-isomer.

The inhibitory action of the stereoisomeric citraconic and mesaconic acids on other organisms, *e.g. B. acidi lactici*, *B. lactis aerogenes*, *B. mesentericus*, *B. cloacae*, and *B. prodigiosus* was also examined and in each case the *trans*-acid was more efficacious than the *cis*-compound.

Zinc hydrogen fumarate was exceptional, however, in being a weaker germicide towards *B. coli* than the corresponding maleate, the latter being inhibitory in concentrations of 1 in 2500.

The germicidal action of dibromo- and *iso*-dibromosuccinic acid has also been studied with the object of throwing light on the relationship of these isomeric acids.

When maleic acid is brominated, *iso*-dibromosuccinic acid is formed, from which by loss of hydrobromic acid bromofumaric acid is obtained. Similarly, fumaric acid forms ordinary dibromosuccinic acid, which finally yields bromomaleic acid.

The inhibitory and germicidal powers of the two acids were determined by the method already described.

	Inhibitory conc.	Germicidal conc.*
Dibromosuccinic acid	1 in 250	1 in 1000
iso-Dibromosuccinic acid	1 in 400	1 in 1550

* Chick-Martin method, using centrifuged culture of *B. coli*.

The *iso*-acid is thus a stronger germicide than dibromosuccinic acid, although it is formed by bromine addition from the original *cis*-acid (maleic) which has been found conversely to be less active than the *trans*-isomeride (fumaric). The results point to a similarity in chemical configuration between maleic and dibromosuccinic acids, and a corresponding similarity between fumaric and *iso*-dibromosuccinic acids, thus confirming the structures ascribed to these acids by chemists.

The fact that the *trans*-acids tend to be more powerful disinfectants than *cis*-acids is of great interest when correlated with Ishizuka's [1897] observation that maleic acid was more poisonous to higher animals than fumaric acid. It is possible therefore that *trans*-compounds may be the starting point for the preparation of a new series of germicides of high bactericidal power, but relatively low toxicity. Fumaric acid itself is a stronger disinfectant than phenol, and substitution of certain groups might still further raise its efficacy. A point of practical significance is the fact that experiments by the authors have shown that bacteria do not acquire a tolerance to the *cis*- and *trans*-acids.

With the object of offering some explanation of the superiority of the *trans*-acids as germicides, experiments were next carried out to study

(i) the effect of the acids on enzyme action,

(ii) their precipitating action on proteins and other colloids,

(iii) their relative absorption by proteins.

Enzyme Action.

(*a*) *Diastase.* Mixtures of saliva, starch solution, and solutions of *cis*- and *trans*-acids were incubated at 40° and the time taken for complete hydrolysis of the starch noted and compared with mixtures of saliva and starch alone without added acid. Iodine was used as external indicator in these experiments. Tests were carried out with maleic and fumaric, also with citraconic and mesaconic acids.

It was found that the *trans*-acids inhibited the hydrolysis of starch to a greater extent than *cis*-acids, thus suggesting a parallel with the bacteriological results.

Similar observations were made in the case of the calcium and zinc hydrogen salts.

The following are the results of a typical experiment:

	Period required for hydrolysis of starch (min.)
Water control	4
Maleic acid (0·05 %)	17
Fumaric acid (0·05 %)	23
Maleic acid (0·03 %)	19
Fumaric acid (0·03 %)	30
Citraconic acid (0·037 %)	8
Mesaconic acid (0·037 %)	21

(*b*) *Pepsin.* The action of the acids on the rate of hydrolysis of proteins by pepsin was investigated by the Mett's tube method. The small rods of coagulated egg-white were immersed in 1 % solutions of pepsin containing varying proportions of the acids, and the lengths of the rods determined after a given period. In this way the extent of digestion could be ascertained.

	Lengths of protein digested by 1 % pepsin	
	8 hours	22 hours
In presence of:		
0·5 % maleic acid	1·7 mm.	4·2 mm.
0·5 % fumaric acid	2·2	4·6
0·5 % citraconic acid	2·0	2·3
0·5 % mesaconic acid	2·8	2·3

The results in general show that the *trans*-acids have a somewhat greater accelerating action on the rate of digestion than the *cis*-acids. This is remarkable in view of the facts that the presence of acid is necessary for digestion by pepsin, and *cis*-acids are stronger acids than the *trans*-isomerides. Evidently the *trans*-acids are again anomalous, their activity not being merely determined by their acidity.

(*c*) *Lipase.* A comparison was next made on the relative action of lipase on triolein and trielaidin, and ethyl esters of maleic and fumaric acids.

The lipase was used in the form of an alkaline extract of ox-pancreas. The trielaidin was prepared by the action of a solution of mercury in nitric acid on olive oil (elaidin test). 2 cc. of olein or the melted elaidin were treated with 1 cc. lipase extract, and one drop of phenolphthalein was added together with sufficient $N/20$ sodium hydroxide to impart a pink tint. The mixtures were kept at 40°. As the fats were hydrolysed, the acids liberated neutralised the alkali, and more $N/20$ alkali had therefore to be added to restore the colour. In the case of the olein, 4 cc. of sodium hydroxide were sufficient, but in the case of elaidin two further additions of alkali were necessary, indicating that olein was hydrolysed less rapidly than elaidin. In view of the greater reactivity of the *trans*-compounds already indicated, the results suggest that elaidin is the *trans*-compound and olein the *cis*-isomeride, a conclusion supported by the melting points of the corresponding acids.

In the case of the diethyl esters of maleic and fumaric acids, 1 cc. of the esters was mixed with 1 cc. of lipase and the mixture incubated at 37° and titrated at intervals with standard alkali. The results were as follows:

	Period of hydrolysis		
Ester	1·5 hours	18 hours	25 hours
	Amount of alkali required for neutralisation		
Ethyl maleate	6·6 cc.	9·3 cc.	11·4 cc.
„ fumarate	5·2	9·4	15·0

It is seen that initially the maleate ester is rather more readily hydrolysed than the fumarate, but as hydrolysis proceeds the *trans*-ester is broken down more rapidly than the *cis*-isomeride.

Protein Precipitation.

The following experiments were suggested by the observation made in the bacteriological work that maleic and fumaric acids caused precipitation of the peptone in the broth tubes, thus rendering the determination of bactericidal power very difficult.

(1) *Aqueous solutions.* Solutions of peptone and various proteins were mixed with solutions of the acids and salts of varying strength, and the effect upon the physical state of the colloids was observed.

Compound	Concentrations required to induce a maximum turbidity or precipitate				
	Peptone %	Mucin* %	Egg-white %	Caseinogen %	Serum %
Maleic acid	0·1	0·19	Acids	>0·4	0·18
Fumaric acid	0·05	0·07	caused	0·4	0·04
Citraconic acid	0·075	—	no precipi-	—	—
Mesaconic acid	0·05	—	tation	—	—
Dibromosuccinic acid	>0·3	0·25	—	—	—
iso-Dibromosuccinic acid	0·3	0·25	—	—	—
Normal zinc maleate	>0·01	>0·07	>0·5	0·15	—
„ fumarate	0·01	0·05	0·5	>0·15	—
Zinc hydrogen maleate	0·01	>0·07	0·5	—	—
„ fumarate	>0·1	0·05	>0·5	—	—
Sodium oleate	0·025	>0·25	>0·75	0·5	—
„ elaidate	0·025	0·25	0·75	0·05	—

* Diluted filtered saliva.

(2) *Precipitation in other media than water.*

(i) Peptone dissolved in anhydrous phenol at 70° was precipitated therefrom by ethyl fumarate, but not by ethyl maleate.

(ii) Lecithin dissolved in ether was more readily precipitated by ethyl maleate.

(iii) Gliadin dissolved in alcohol was more quickly precipitated by the zinc maleates than by the fumarates. In the case of the calcium salts, the *trans*-compound was the more active precipitant.

A consideration of the tabulated results shows that almost consistently the *trans*-compounds are more efficacious precipitants than the *cis*-isomers, thus bringing out a very interesting parallel with the bacteriological observations. The exceptions to this generalisation are very few, viz. the acid zinc salts of maleic and fumaric acids in precipitating peptone, gliadin and egg-white, and precipitation of lecithin by the ethyl esters.

Furthermore, in the case of serum-proteins, not only does fumaric acid cause precipitation more readily than maleic acid, but it also induces a redispersal of the precipitated proteins in lower concentration. Dispersion of the coagula is thus not determined merely by the strength of the acid, but, like precipitation, is bound up in the configurational difference of the isomeric acids.

In a recent paper on coagulation of proteins by organic acids Isgarischev and Bogomolova [1926] have also shown that fumaric acid is more active than maleic in precipitating caseinogen.

It is of interest to note that in the case of peptone *iso*-dibromosuccinic acid is more efficacious as a precipitant than its isomer, this being consistent with the bacteriological results.

Imbibition.

It is well known that gelatin possesses the property of taking up water to a considerable extent when immersed therein, and that this imbibition is increased enormously when small quantities of acids or bases are dissolved in the water. Maleic and fumaric acids were therefore investigated with respect to this property. The technique was as follows.

Strips of gelatin weighing about 0·2 g. were immersed in 20 cc. of solutions of maleic, fumaric and other acids for a definite length of time. They were then removed, the surface dried with filter paper, and weighed again. The increase in weight showed the imbibition, which was then expressed as a percentage of the original weight of gelatin. The following example gives the results of one experiment.

The gelatin was allowed to remain in the acid solutions for 42 hours.

Acid	%	Weight of gelatin g.	Gain in weight g.	Percentage imbibition
Maleic	0·2	0·21	5·16	2500
,,	1	0·20	4·12	2050
Fumaric	0·2	0·20	4·31	2100
,,	0·5	0·20	5·76	2850
Dibromosuccinic	0·2	0·20	3·62	1800
,,	1	0·20	3·38	1700
Succinic	0·2	0·20	2·52	1250
,,	1	0·20	4·66	2300
Citraconic	0·2	0·20	4·81	2400
,,	1	0·20	4·96	2450
Mesaconic	0·2	0·20	4·32	2150
,,	1	0·19	5·52	2900
Itaconic	0·2	0·20	3·26	1600
,,	1	0·19	5·08	2650
Water		0·20	1·36	660

The use of 0·5 % fumaric acid instead of 1 % was necessitated by its limited solubility.

The above results show that in the more dilute solutions the *cis*-acids caused the greater imbibition, but that in the case of stronger solutions this was reversed, the *trans*-acids having more effect.

This change with rising concentration holds also in other cases. Thus not only do the *cis*- and *trans*-acids reverse their effects, but while in diluter solutions the saturated acid (succinic) has less effect than the unsaturated one (maleic), in the higher concentrations it causes greater imbibition. (It must be noted, however, that succinic always has less effect than fumaric acid.) Itaconic acid is less effective in increasing imbibition than the *cis*- and *trans*-isomerides (citraconic and mesaconic acids) in dilute solutions, but in stronger concentrations there is little difference between the three acids.

To study the variation of imbibition with concentration, ranges of concentrations from 0·1 % to 0·5 % for maleic and fumaric acids and 0·1 % to 1 % for citraconic and mesaconic acids were used, with results embodied in the following table:

Percentage imbibition.

Concentration %	Maleic acid	Fumaric acid	Concentration %	Citraconic acid	Mesaconic acid
0·1	4800	4700	0·1	4850	4600
0·2	4550	4500	0·3	4550	4800
0·3	4100	5000	0·5	—	4750
0·4	4050	4500	0·7	4100	4650
0·5	3500	5050	1	3600	4900

It is seen that, while the *cis*-acid in each example caused a steady diminution of imbibition with increase of concentration, the *trans*-acid gave irregular results, although imbibition tended on the whole to increase as in former experiments.

The effect of the *cis*- and *trans*-acids on imbibition was studied in the first place with the object of throwing light on the conditions of protein precipitation. The results, although of interest, are of some complexity, and it is difficult at the present time to understand their real significance. It is remarkable that the *trans*-acids, although the weaker acids, again behave anomalously in increasing imbibition under certain conditions to a greater extent than the *cis*-compounds, and for this reason it may be considered likely that the process of imbibition is involved in the mechanism of disinfection and precipitation.

In a recent paper Isgarischev and Pomeranzeva [1926] have shown that maleic acid increases the imbibition of water by caseinogen to a greater extent than fumaric. The foregoing results obtained with gelatin are, however, more complex, and indicate that *trans*-acids sometimes induce greater imbibition.

The distribution of *cis*- and *trans*-acids between water and protein.

The object of the following experiments was primarily to ascertain in what degree the germicidal and precipitating power of the *cis*- and *trans*-acids depended on the relative absorption by, or chemical action with, the bacterial proteins. The method adopted was to allow solutions of the acids free access to a protein, so that the acids could distribute themselves between the water and the protein phases, the changes in concentration in the one phase (water) being followed by titration methods.

In connection with this work the investigations of Hermans [1924] are interesting. His experiments were concerned with the absorption of *cis*- and *trans*-isomers by charcoal and, using maleic and fumaric acids, he found the former to be the less absorbed. He also showed that if the acids were dissolved in dilute (0·05 %) HCl, the relative absorbabilities were unaffected, although the hydrogen ion concentrations were now equal. Hence the strength of the acids, maleic being the stronger, does not determine the relative extent of their absorption.

The procedure in the protein experiments was as follows:

1 g. of gelatin, in strips, was immersed in a solution of each acid and allowed to remain at room temperature therein until equilibrium had been reached.

The length of time required for equilibrium to be established was determined by preliminary titration experiments, and it was found that 48 hours was easily sufficient.

The influence of concentration upon the absorption of acids by gelatin.

1 g. gelatin in 100 cc. solution of acids.

Acid	Initial concentration %	Final concentration %	Amount of acid absorbed %	Distribution ratio. Amount of acid held by 1 g. protein. / Amount in 1 g. water
(1) Maleic	2·38	2·20	0·18	8
	1·00	0·88	0·12	14
	0·44	0·38	0·06	16
	0·087	0·044	0·04	91
Fumaric	0·50	0·46	0·04	9
	0·10	0·044	0·05	114
(2) Maleic	0·99	0·93	0·06	6
	0·51	0·42	0·09	22
	0·20	0·14	0·06	43
	0·099	0·058	0·04	70
Fumaric	0·47	0·44	0·03	7
	0·19	0·16	0·03	19
	0·095	0·064	0·03	47

A survey of these results indicates that in the case of maleic acid, the amount of acid absorbed by the gelatin increases absolutely, but not relatively, with ascending concentration, thus causing an actual diminution in the value of the distribution ratio. This relationship is suggestive of an adsorption process.

In the case of fumaric acid, however, the amount taken up is practically constant at all concentrations. Furthermore, it will be noticed that the *cis*-acid is absorbed in greater amount than the *trans*-acid, the results thus being exactly opposite to those obtained by Hermans [1924] in the case of charcoal.

Further experiments with other acids also bring out this point, the same technique being employed as before.

Acid	Distribution ratio
Maleic	14
Fumaric	10
Citraconic	20
Mesaconic	14
Succinic	7
Dibromosuccinic	12
iso-Dibromosuccinic	5

It is seen that the *cis*-compounds are consistently absorbed to a greater extent than the *trans*-isomerides, although the *trans*-derivatives were previously found to be the more efficacious as germicides and protein-precipitants. Bactericidal and precipitating action are thus not determined merely by selective absorption of the agent by the proteins, but are intrinsic properties independent of solubility or adsorption.

Work was next put in hand to ascertain whether any evidence could be obtained of a chemical action between the unsaturated acids and amino-acids or proteins.

The experiments with maleic acid and gelatin pointed to adsorption, but in the case of fumaric acid the amount absorbed was constant over a range of concentration, suggestive of chemical action. There was a possibility that amino-groups in either amino-acids or proteins could add on at the double-linkage of the unsaturated acids thus:

$$\begin{matrix} CH.COOH \\ \| \\ CH.COOH \end{matrix} + NH_2.CH_2.COOH = \begin{matrix} CH_2.COOH \\ | \\ CH.COOH \\ | \\ NH.CH_2.COOH \end{matrix}$$

By titrating amino-acids, *e.g.* glycine with standard alkali in the presence of either maleic or fumaric acids, analogously to Sørensen's formalin method, no evidence, however, could be obtained of such a reaction. The amino-groups of gelatin were next substituted by various methods, and the effect of this change upon the absorptive capacities of the proteins for the acids was studied.

The following products were employed.

1. Gelatin treated with formalin for 2 days

$$- NH_2 + H.CHO = - N = CH_2 + H_2O.$$

2. Gelatin treated with 0·25 % benzoquinone solution for 2 days, aromatic nuclei being thereby introduced into the NH_2 groups.

3. Desamino-gelatin was obtained by treating gelatin with nitrous acid, the NH_2 groups being replaced by OH.

In all cases the protein was finally washed in running water and dried at 100° before use.

The absorption of maleic and fumaric acids by the foregoing materials, with ordinary gelatin as control, was next determined by the methods already described.

	Distribution ratios	
	Maleic acid	Fumaric acid
Gelatin	3	2
Formalised gelatin	12	8
"Quinone" gelatin	16	12
Desamino-gelatin	10	10

A consideration of the figures shows that in nearly every case the *cis*-acid is taken up by the protein to a greater extent than the *trans*-compound, thus confirming earlier observations. Moreover, by introducing substituents into the amino-groups of gelatin, and even replacing them by hydroxyl groups (as in the desamino-product) absorptive power of the protein for the two acids is actually in all cases considerably increased. There is thus no evidence of chemical action between the unsaturated acids and the amino-groups of proteins or glycine. In the case of maleic acid, the uptake would appear to be of the nature of adsorption, and in the case of fumaric acid adsorption is probably the main factor, although it happens that a maximum uptake is indicated at an unusually low concentration.

SUMMARY.

The dibasic unsaturated *trans*-acids are superior to the *cis*-isomerides in bactericidal action, although they are weaker acids. Their germicidal power is therefore not determined by hydrogen ion concentration, but must depend upon the stereochemical configuration of the molecule.

The *trans*-compounds are also generally more efficacious as protein-precipitants and more reactive towards enzymes than the *cis*-isomers. Notwithstanding, the *cis*-acids are absorbed by protein in larger amount than the *trans*-acids from aqueous solution.

The higher bactericidal power of the *trans*-compounds is therefore apparently due not to their selective absorption by the bacterial proteins, but may be associated with their greater precipitating action on proteins, or inhibitory effect upon enzymes.

The uptake of the *cis*- and *trans*-acids by protein is probably an adsorption process, and there is no evidence of chemical interaction with protein or amino-acid by addition of amino-groups through the unsaturated linkage.

The observations bring out very clearly the analogy between *cis*- and *trans*-compounds, and *ortho*- and *para*-isomers in the aromatic series. In each case the more symmetrically constituted compounds (*trans*- and *para*- respectively) are in general the more powerful germicides and precipitants.

Finally, the results suggest a problem of practical interest. Whilst fumaric acid is a weaker poison than maleic acid to the higher animals, it is conversely a stronger disinfectant. As fumaric acid is more bactericidal than phenol, *trans*-derivatives may thus be useful as germicides, possessing relatively low toxicity.

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CXXXVI. THE INFLUENCE OF TEMPERATURE ON THE p_H OF BLOOD.

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The effect of temperature on the p_H of blood has been the subject of controversy. The discrepancy has arisen from the fact that in the particular technique employed by an observer, sometimes the pressure of CO_2, sometimes the concentration and sometimes neither was maintained at the same level at the two temperatures.

The easiest observation to make is one in which the CO_2 pressure is the same whilst the temperature is varied. This was the case in Hasselbalch's [1917] experiments upon ox blood. He found, somewhat to his surprise, that the p_H calculated both from electrometric observations and from the proportion of CO_2 in the hydrogen used and the combined CO_2 in the blood, was the same at 18° as at 38°. Michaelis and Davidoff [1912], on the other hand, in their early observations, found the p_H 0·21 higher at 38° than at 18–20°. Corral [1921] obtained results similar to Michaelis, viz. 0·23 difference in p_H.

In Michaelis and Davidoff's method the blood is enclosed in a stoppered U-shaped electrode vessel and shaken with a bubble of hydrogen. If the bubble of hydrogen be small compared with the volume of the blood, the content of CO_2 in the latter remains approximately constant whatever the temperature, whereas in Hasselbalch's later technique the blood is equilibrated with the same CO_2 pressure at different temperatures and the content varies in accordance with the solubility of CO_2 at those temperatures.

As a matter of fact, the content of CO_2 is not constant in Michaelis' method of procedure because the volume of hydrogen is not reduced to negligible proportions but, as the buffering effect of the $NaHCO_3$ and haemoglobin is considerable, nearly the same p_H is found as if it were.

The conditions in Corral's experiments were intermediate. He used Hasselbalch's older form of electrode vessel and, if we understand him aright, the volume of hydrogen was about equal to the volume of blood.

Effect of temperature on p_H of blood when the pressure of CO_2 is maintained constant.

We agree with Hasselbalch that, when the CO_2 *pressure* is constant and the CO_2 *content* varies with the changing solubility of CO_2 in water, the effect of temperature on the p_H of blood is very slight.

In the following observations the details of the electric measurements were those outlined in a previous communication to this *Journal* [Lepper and Martin, 1926]. The blood was reduced and equilibrated with H_2 containing about 5·8 % CO_2 and the same mixture of hydrogen and CO_2 was used in the electrode vessel. The arrangements for doing this in a thermostat were similar to those described by Warburg [1922]. An abundant supply of hydrogen with the desired mixture of CO_2 was compressed into a steel bottle and could be turned by a three-way cock either into the rotating bottle in which the blood was brought into equilibrium with the gaseous mixture or into the electrode. Blood could be drawn from the bottle into the electrode or *vice versa* without coming into contact with air.

	p_H at 18°	p_H at 38·5°
Rabbit's blood containing 54 % haemoglobin, sodium fluoride 0·02 % and potassium oxalate 0·04 %	7·420	7·427
Do. Do.	7·391	7·385
Human blood with same amount of oxalate and fluoride	7·370	7·364

The difference in p_H at 18° and 38° is within the error of our experiments. When freshly drawn blood was used we found it to be essential to prevent glycolysis occurring as this happens sufficiently rapidly at 38° to vitiate the results. Hasselbalch, we believe, avoided this source of error by using blood which had been whipped and had stood some time. We did so by adding sodium fluoride, which Evans [1922] found to inhibit glycolysis.

As, with constant CO_2 pressure, the p_H of blood remains nearly the same notwithstanding the rise in temperature, the fall in acidity due to diminished CO_2 concentration in the liquid at the higher temperature must be compensated by the increase in the dissociation of the weak acids, H_2CO_3 (first H), H_3PO_4 (second H), and proteins. Raising the temperature of 0·03 *M* $NaHCO_3$ solution in equilibrium with 5 % CO_2 from 18° to 38° decreased the concentration of CO_2 dissolved to 0·6 of its former value. This, by its effect on the $CO_2/NaHCO_3$ ratio would raise the p_H by 0·22. As a matter of fact, the p_H is raised by only about half this amount, half being compensated by the increase in the dissociation of H_2CO_3.

In the case of blood, with its other weak acids, phosphates and proteins, the influence of temperature upon their various acid dissociation constants comes also into play. The problem is a complicated one and cannot be entered into here, but it is obvious that the net effect, on warming the solution, on the different dissociation coefficients, approximately balances the effect of diminished CO_2 content, provided the excess of CO_2 dissolved and the amount driven out of the $NaHCO_3$ by the increased acidity of the proteins, etc., can leave the system.

This may not be merely a chance happening but a provision against alteration of p_H with change of temperature of the blood in different parts of the circulation.

Effect of temperature on the p_H of blood when the concentration of total CO_2 is maintained constant.

The effect of raising the temperature, the total CO_2 concentration remaining constant, is to lower the p_H of solutions containing a mixture of CO_2 and $NaHCO_3$. Hastings and Sendroy [1924] found for 0·033 *M* $NaHCO_3$ a fall of 0·055 for 18° rise in temperature in the presence of 0·1 *M* NaCl. We have found about the same fall, 0·057 for 20°, for 0·02 *M* $NaHCO_3$ in presence of 0·13 *M* NaCl.

The fall in p_H is considerably greater with plasma or blood. In both cases, the influence of temperature on the dissociation of the proteins is also manifested and this effect should, from Meyerhof's [1922] determination of the heat of ionisation of serum-albumin, be considerable.

We have not been able to arrange a satisfactory experiment to determine the effect of temperature under the above conditions by means of the hydrogen electrode. Our efforts with the early method of Michaelis and Davidoff [1912] afforded discordant results as the blood is not de-oxidised. Various attempts to obviate this were not very successful. On one occasion we determined the p_H of a sample of blood electrometrically at 38° and then drew it off from the electrode vessel over mercury and allowed it to cool to 18° and subsequently expelled it into a tube under paraffin and stoppered the tube. We then sedimented the corpuscles by centrifuging and determined the p_H of the plasma with the quinhydrone electrode. For the latter determination, we used the method described by Cullen and Biilmann [1925]. The p_H at 38° was found to be 7·24; at 18° it was 7·44 and 7·48 respectively in two different samples, a mean difference of 0·22. The agreement between the duplicate observations is not good but the quinhydrone electrode is not suitable for accurate determinations of c_H in solutions so alkaline as p_H 7·4.

It then occurred to us that the problem could be solved by using the modified colorimetric method of Cullen [1922] described in our previous paper [Martin and Lepper, 1926], in which the diluted plasma or blood is hermetically sealed in a glass tube so that the CO_2 cannot escape.

Cullen found a difference of about 0·2 between the p_H of plasma at 20° and 38°. The p_H was arrived at electrometrically at 38° and colorimetrically at 20° so that any discrepancy between these two methods falls upon the determination.

As in our micro-method the plasma or blood is in hermetically sealed tubes with only a minute amount of gas in the capillary to permit of sealing, the effect of temperature on the colour of the phenol red in the diluted plasma can be observed very simply by warming or cooling it. Determinations at both temperatures are in this case made on the identical sample and by the same method. It is necessary to apply the appropriate corrections for the influence of temperature on the dissociation of the indicator to arrive at the real p_H at the higher temperature.

Observations on five samples of plasma are set out in Table I below. The procedure was that already described [Martin and Lepper, 1926, p. 38]. Each tube was immersed in a test-tube of water with a slip of filter paper in it as a background. The apparent p_H of the diluted plasma was determined firstly at 18°, then at 38° and lastly again at 18°.

Table I.

Apparent p_H of plasma at			Mean difference
18°	38°	18°	between 18° and 38°
7·54	7·47	7·52	0·06
7·55	7·50	7·56	0·06
7·58	7·50	7·57	0·07
7·60	7·52	7·60	0·08
7·58	7·52	7·58	0·06
		Mean	0·066

Hastings and Sendroy [1924] found that the p_H for phenol red increased 0·13 between 20° and 38°, so that 0·14 should be deducted from the apparent p_H at 38°. This gives 0·206 as the amount to be subtracted from the p_H found at 18° to arrive at the p_H of human plasma at 38°. This is in close agreement with Cullen's figures [Cullen, 1922].

The effect of temperature on the p_H of diluted blood can be determined in the same way. Hasselbalch [1917] found that when the CO_2 pressure was constant the influence of temperature upon the p_H of blood was quite different from that on serum. He found serum behaved as $NaHCO_3$ sol. We do not quite agree with this statement, but grant that it reacts more like a weak $NaHCO_3$ sol than like blood, so it would appear unlikely that the plasma and blood would behave alike when the total CO_2 content is maintained constant. Indeed, considering the high concentration of haemoglobin in blood, this seemed to us improbable.

The direct measurements of Brown and Hill [1923] give − 10,500 cals. as the heat of ionisation of haemoglobin, which is of the same order of magnitude as Meyerhof [1922] arrived at from their heats of neutralisation for serum-albumin and serum-globulin. The acid dissociation constants of haemoglobin should, therefore, according to the van 't Hoff isochore

$$\frac{d}{dT}(\log k) = \frac{10{,}500}{RT^2} \text{ or its integral}$$

$$k_{18}/k_{37} = e^{\frac{10{,}500\,(T_{18} - T_{37})}{1{\cdot}98 \times T_{18}T_{37}}}$$

be doubled when the temperature rises from 18° to 38°.

We have ascertained the effect of temperature on the p_H of blood by studying the influence of the temperature at which the corpuscles are separated upon the p_H of the resulting plasma. We employed the micro-method described above. Fluoride is essential for this experiment. Without it the tubes become progressively acid at 37°. Six tubes of blood diluted 1 in 15 with saline containing sodium fluoride and indicator were prepared and hermetically sealed.

Two of them were centrifuged in a room the temperature of which was 19° and the p_H of the plasma determined at 19°. Four others were placed in water at 37° for half an hour and centrifuged in a room, the temperature of which was 37°. They were replaced in a bath at 37° and their p_H determined whilst at this temperature. These four tubes were now allowed to cool and the corpuscles were redistributed throughout the tubes by shaking. After 15 minutes at 19° they were centrifuged at 19° and their p_H determined at 19°. The results are shown in Table II below.

Table II.

	Apparent p_H at 37°. Corpuscles separated at 37°	p_H at 19°. Corpuscles separated at 19°	Difference
1	—	7·58	—
2	—	7·60	—
3	7·54	7·60	0·06
4	7·51	7·58	0·07
5	7·51	7·58	0·07
6	7·53	7·59	0·06
			0·065

When 0·13 is added for the dissociation of the indicator to the mean difference of 0·065, the real difference of p_H of the diluted blood for 18° increase in temperature is 0·195. This, for 20° difference, would be 0·216. This is a little more than we obtained with plasma, which was 0·206 for 20°. The difference is not, however, outside the experimental errors.

When CO_2 cannot escape from the system, the effect of temperature on the p_H of plasma and corpuscles seems to be very nearly the same, viz. a fall of about 0·2 for a rise of temperature from 18° to 38°. This, to us, unexpected result means that an adjustment of electrolytes between corpuscles and plasma is not necessitated as the temperature of the blood alters in circulating through the body.

Conclusions.

1. As Hasselbalch found to be the case with ox-blood, if the *pressure* of CO_2 remains constant, the p_H of human and rabbit blood does not alter significantly when the temperature varies from 18° to 38°.

2. With *concentration* of total CO_2 constant, the p_H of human blood falls about 0·2 when the temperature is raised from 18° to 38°.

3. The effect of temperature, with concentration of total CO_2 constant, is the same upon the c_H of the corpuscles and the corresponding plasma.

4. If 0·2 be deducted from the p_H of the plasma determined by the colorimetric method of Cullen or Hawkins [1923] or by the authors' micro-colorimetric method at 18°, it will give the p_H of the corresponding blood at 38°.

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CXXXVII. OBSERVATIONS ON THE EXTENT TO WHICH THE ELECTROMETRIC DETERMINATION OF THE [H·] OF BICARBONATE SOLUTIONS IS INTERFERED WITH BY THE PRODUCTION OF FORMIC ACID AT THE ELECTRODE.

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Evans [1921], when comparing the [H·] of bicarbonate solutions and of blood as estimated colorimetrically (dialysis method of Dale and Evans [1921]), with that obtained by the hydrogen electrode, found the p_H to be consistently about 0·2 lower by the latter method. He attributed this difference to the production of formic acid at the electrode and concluded that it was impossible to obtain the correct p_H of a bicarbonate solution electrically.

Formic acid is readily oxidised to CO_2 and H_2O in the presence of platinum black. Wieland [1912] showed that under suitable conditions the reverse reaction occurs and that a small quantity of formic acid is formed when water saturated with CO_2 and hydrogen at a pressure of one and a half atmospheres is kept in presence of palladium black; the amount produced in 20 cc. of the solution of CO_2 was only a few milligrams.

Bredig and Carter [1914] by increasing the pressure of hydrogen to 60 atmospheres, working at a temperature of 70° and using sodium bicarbonate to fix the formic acid produced in the presence of palladium black obtained formate amounting to 75 % of the bicarbonate present. They patented the process.

Evans had therefore good reason to suppose that traces of formic acid might be produced at the hydrogen electrode when working with solutions containing bicarbonates.

Warburg [1922, p. 248] criticised Evans' conclusions on several grounds. He also says: "It can easily be shown that the combined CO_2 in an alkali bicarbonate solution does not decrease when treated for an hour with hydrogen in presence of platinum black." Warburg does not mention the concentration of his bicarbonate solutions nor how he determined the combined CO_2.

We have elsewhere [1926] published observations showing that there is not a discrepancy such as Evans supposed between colorimetric and electrometric determinations of p_H of bicarbonate solutions provided that the

concentration of the Na is the same as that in the standard phosphate solutions. As the concentration of Na in blood-serum and Sørensen's $M/15$ phosphate solution of p_H 7·4 is approximately the same, colorimetric and electrometric observations should agree and Cullen and Hastings [1922], Warburg [1922], Verney and Bayliss [1923, 1926], Lepper and Martin [1926], found this to be the case. Nevertheless, we believe Evans was correct in concluding that some reduction of CO_2 to formate does occur at the electrode, although the amount of formic acid formed is insufficient to account for the discrepancy between his colorimetric and electrometric results. It can only be appreciated in very dilute solutions, *e.g.* less than 0·005 M $NaHCO_3$, but its action limits the correct determinations of c_H to solutions above this concentration.

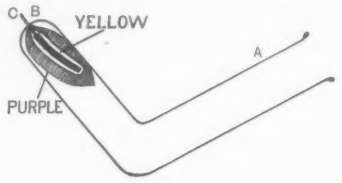

Fig. 1.

Working with a bicarbonate solution such as 0·0002 M $NaHCO_3$ shaken with room air which affords a solution of CO_2 of about 0·000014 M and phenol red as indicator, the production of acid at the electrode can be observed in the following ways. Our first experiments were made with an electrode vessel made of a piece of hard glass tubing, AB, bent at an angle and closed at end B (Fig. 1). The electrode, C, was thickly coated with platinum black. After charging the electrode with hydrogen the tube was completely filled with the bicarbonate solution containing 0·001 % phenol red; the p_H was about 7·1. The bicarbonate solution in immediate contact with the freshly charged electrode became lemon yellow and for 2 mm. just outside this the full purple colour of phenol red in alkaline solution developed. If the electrode vessel was kept undisturbed the purple streamed down into the bend of the tube.

The explanation of these appearances seemed to be that CO_2 was adsorbed from the bicarbonate solution by the platinum black, leaving it more alkaline for a layer of 2 mm. surrounding the electrode. Simultaneously the film immediately in contact was rendered more acid by reduction of the adsorbed CO_2 to formic acid. The effect is not due to a specific action of the electrode on phenol red, as the production of acid could be equally well shown if neutral red were used, a substance having an entirely different chemical composition from that of phenol red.

As mentioned above, the appearances could be most easily seen when a 0·0002 M solution of bicarbonate shaken up with room air was used. With this concentration they persisted for some hours. If 0·01 M bicarbonate solution saturated with 2·4 % CO_2 were used the purple zone was narrower and only seen for a few minutes after the fluid came in contact with the electrode.

To test our interpretation we made the following experiment. An electrode *A* (see Fig. 2) made of a circular disc of platinum 2·5 cm. in diameter and 0·25 mm. thick was mounted on a piece of glass tubing, *B*. The electrode was more thickly coated with platinum black on one surface, *a*, than on the other, *b*. A cylinder of hard glass, *E*, into which the electrode exactly fitted was selected and furnished with a rubber stopper pierced by two holes. Through one of these the glass tubing of the electrode was passed and adjusted so that when the cork was in position the platinum disc was two-thirds way down the cylinder. In the other hole, *G*, a piece of glass tubing of about 2 mm. internal bore was inserted. The electrode was charged with hydrogen by making it the cathode in 10 % sulphuric acid.

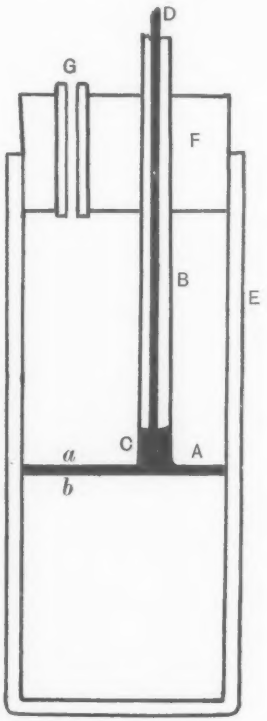

Fig. 2.

The cylinder was filled to the top with 0·0002 *M* $NaHCO_3$ containing 0·001 % phenol red and saturated with room air containing 0·035 % CO_2. The resultant p_H was about 7·2 and the colour orange. The electrode was taken out of the acid, washed with distilled water for about a minute and placed in the cylinder. Sufficient washing with distilled water will secure the

"neutrality"[1] of an electrode previously saturated with hydrogen. The stopper having been pushed home the cylinder was inverted and hydrogen introduced by a capillary tube through *G*. The platinum disc was a sufficiently good fit to permit of inverting the tube without any liquid escaping between it and the glass so that the hydrogen only came in contact with one surface, *a*, of the electrode. After waiting 5 minutes for this surface to become charged with hydrogen the cylinder was placed right way up. The object of these manœuvres was to secure that one surface, *a*, of the electrode should be saturated with H_2 whereas the other surface, *b*, should be neutral, *i.e.* impregnated with neither hydrogen nor oxygen.

After a few minutes a deep purple colour developed in the fluid in immediate contact with surface *b* and a lemon-yellow colour appeared in the fluid in contact with surface *a*. The platinum disc fitted the cylinder exactly so that diffusion was minimised and the two phases of the reaction could be observed side by side without interfering with each other. The changes of colour spread slowly out from each surface of the electrode until, after 2 hours, all the fluid in the lower half of the cylinder was purple and that in the upper half lemon yellow.

We explain these appearances as follows: both surfaces of the disc adsorbed CO_2; as surface *b* was neutral no further reaction took place and the fluid in contact with it became alkaline. At surface *a*, however, owing to the presence of H_2 in the platinum black the CO_2 adsorbed was reduced to formic acid and the solution in contact with *a* became acid.

Reduction of CO_2 at the electrode does, we believe, limit the use of the hydrogen electrode when dealing with very weak solutions of bicarbonate. The following calculations of p_{K_1}, at 18° from our determinations of the [H·] of bicarbonate solutions of diminishing concentration show how its value slowly rises from increased activity of the hydrogen ions and then suddenly falls away between 0·002 and 0·0002 *M*. This we attribute to the formation of formic acid at the electrode becoming significant at the low concentration of bicarbonate.

Influence of concentration of $NaHCO_3/CO_2$ on p_{K_1}.

$NaHCO_3$ *M*	Pressure of CO_2 mm.	*M* CO_2	p_H electrometrically	p_{K_1}
0·02	25·0	0·00135	7·48	6·31
0·01	18·5	0·00101	7·32	6·32
0·002	18·5	0·00101	6·66	6·36
0·00002	0·26	0·000014	7·00	5·85

[1] By "neutrality" is here meant an electrode which is charged neither with H_2 nor O_2. If washed too long with distilled water it becomes an O_2 electrode.

Conclusions.

1. The production of an acid at the hydrogen electrode in bicarbonate solutions can be demonstrated when the concentration of bicarbonate is small by adding an indicator to the solution. Under these circumstances an acid zone can be seen immediately surrounding the electrode, enveloped by a wider alkaline zone due to adsorption of CO_2 by the platinum black.

2. This production of acid does not, however, significantly affect the determination of the p_H of bicarbonate solutions until the molecular concentration is less than 0·005 *M*.

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CXXXVIII. THE CHEMICAL CONSTITUTION OF SPERMINE.

III. STRUCTURE AND SYNTHESIS.

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THE formula for spermine, isolated from animal organs and identical with the base occurring in human semen [Rosenheim, 1924], was established as $C_{10}H_{26}N_4$ by the work reported in the first of this series of communications [Dudley, M. C. Rosenheim and O. Rosenheim, 1924]. Carefully controlled oxidation of the base with hydrogen peroxide and with potassium permanganate failed to produce evidence of any resistant ring structure in the molecule; spermine does not reduce permanganate in faintly acid solution and displays no optical activity in the polarimeter. These observations and the hydrogen content justify the inference that it is a saturated aliphatic base which does not contain any asymmetric carbon atoms, assuming that no racemisation has occurred during its isolation.

The action of nitrous acid on spermine is complicated and in Van Slyke's apparatus yields indefinite results similar to those obtained with the aliphatic diamines. By the interaction of silver nitrite and spermine hydrochloride crystalline products are formed, but the reaction, on account of its complexity, was unlikely to throw direct light on the constitution of the base and was therefore not followed up: for the same reason the study of the action of magnesium on the chloroaurate was abandoned. It is of interest to note that the free base spermine, when pure, does not possess the characteristic odour with which it is usually associated, but that a volatile base with this property is produced both by the oxidation of the pure substance in all the above-mentioned reactions and also during the decomposition of the chloroaurate.

It has already been reported [Dudley and Rosenheim, 1925] that, on exhaustive methylation of spermine, ten methyl groups are taken up. This fact, together with the observation that spermine forms a tetrabenzoyl derivative, establishes the presence in its molecule of two NH-groups and two NH_2-groups: the four nitrogen atoms are thus accounted for.

The final clues to the constitution of the base were provided by an examination of the degradation products of the quaternary base, decamethylspermine, and of those resulting from destructive distillation of spermine hydrochloride. Among the products of the latter process pyrrolidine was identified and this suggested the presence in spermine of the chain N—C—C—C—C—N, since this same base is formed when the hydrochloride of tetramethylenediamine (putrescine) is destructively distilled.

Information concerning the remaining six carbon atoms was supplied by the degradation products obtained by distilling the sulphide of decamethylspermine. This salt was chosen in preference to the chloride since Clarke [1913] had found that by distilling the sulphide of the quaternary base

$$(CH_3)_3.OH.N.[CH_2]_4.N.OH.(CH_3)_3$$

excellent yields of the corresponding tertiary base resulted, whilst Willstätter and Heubner [1907], using the chloride, obtained only trimethylamine and methylpyrrolidine.

It seemed therefore advisable, in view of the complexity of decamethylspermine and the probable presence in it of the $>N.[CH_2]_4.N<$ group, to distil that salt which was less likely to give rise to ring compounds.

The main products obtained by the degradation of decamethylspermine sulphide were two basic fractions, one with a much higher boiling-point than the other. The more complex high-boiling base proved to be the tertiary base hexamethylspermine which of course was of no value in elucidating the structure of spermine; but from the lower-boiling basic fraction another tertiary base, tetramethyltrimethylenediamine $(CH_3)_2.N.[CH_2]_3.N.(CH_3)_2$ was isolated and identified[1]. This suggested the presence in spermine of the chain N—C—C—C—N.

The remaining six carbon atoms were therefore accounted for on the assumption that they consisted of two groups of three, each between two nitrogen atoms.

The two following formulae would, therefore, be in accordance with these analytical findings and all previously observed properties of spermine:

(*a*) $NH_2.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.NH_2$;

(*b*) $NH_2.CH_2.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.NH_2$.

It was decided to test these two possibilities by synthesis.

The synthesis of (*a*) was accomplished by condensing $\alpha.\delta$-diaminobutane (putrescine) with excess of α-phenoxy γ-bromopropane. Treatment of the resulting compound, $\alpha.\delta$-bis[γ'-phenoxypropylamino]-butane, with hydrobromic acid resulted in the replacement of the two phenoxy-groups by bromine and the action of ammonia on this dibromide gave the compound (*a*).

[1] Some of the data recorded on pp. 1088 and 1089 suggest that this fraction may also have contained a small amount of tetramethyltetramethylenediamine derived from the tetramethylenediamine moiety of the spermine molecule.

The synthesis is represented by the following reactions:

$$\underset{(\alpha.\delta\text{-Diaminobutane})}{NH_2.CH_2.CH_2.CH_2.CH_2.NH_2} + \underset{(\alpha\text{-Phenoxy-}\gamma\text{-bromopropane})}{C_6H_5O.CH_2.CH_2.CH_2.Br\ (\text{excess})}$$

$$\longrightarrow \underset{(\alpha.\delta\text{-Bis}[\gamma'\text{-phenoxypropylamino}]\text{-butane})}{C_6H_5O.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.OC_6H_5}$$

$$\xrightarrow{HBr} \underset{(\alpha.\delta\text{-Bis}[\gamma'\text{-bromopropylamino}]\text{-butane})}{Br.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.Br}$$

$$\xrightarrow{NH_3} \underset{(\alpha.\delta\text{-Bis}[\gamma'\text{-aminopropylamino}]\text{-butane})}{NH_2.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.NH_2}$$

This compound proved to be in all respects identical with spermine. It gave the characteristic insoluble phosphate, and comparison of this and other salts with the corresponding compounds prepared from spermine which had been isolated from animal organs revealed complete identity between the synthetic and the natural bases.

The constitution of spermine is therefore established as $\alpha.\delta$-bis[γ'-aminopropylamino]-butane.

EXPERIMENTAL.

I. Destructive distillation of spermine hydrochloride.

The usual procedure for the separation of the reaction products was followed; namely, removal of ammonia as the chloroplatinate and subsequent conversion of the remaining bases into chloroaurates and picrates.

Evidence obtained in preliminary experiments suggested the presence of methylamine as well as of ammonia in the platinum fraction and of pyrrolidine and *N*-methylpyrrolidine in the gold fraction. Since the absence of *N*-methyl groups from spermine itself could be assumed from the negative result of an attempted determination by Hertzig and Meyer's method, the isolation of the methylated bases would obviously not contribute any additional information as to the constitutional formula. Only two experiments therefore are here reported, the first leading to the identification of ammonia and pyrrolidine and the second demonstrating the probable presence of *N*-methylpyrrolidine.

(*a*) *Identification of ammonia and pyrrolidine.*

8 g. spermine hydrochloride were heated, in quantities of 0·5 to 1 g., in small sublimation tubes to 310°, at which temperature a cloud of subliming crystalline material was observed. The temperature of the bath was raised to 330° and held there for 20 minutes when the decomposition was apparently complete. The sublimate consisted of white crystals mixed with purplish brown, oily liquid. Some charred material remained behind which was much less in amount if the reaction was carried out in a current of CO_2[1]. 4·5 g. of the spermine hydrochloride were decomposed by this modified method, the remainder by simple heating.

[1] It was found later that charring was almost completely avoided when the operation was conducted *in vacuo*.

The contents of the tubes were dissolved in dilute HCl, filtered, made alkaline and steam-distilled. The cloudy distillate, containing a small quantity of an oil, was rendered faintly acid by addition of HCl. The oil dissolved and the clear solution was concentrated to about 120 cc. when the addition of 8 g. platinum chloride in 20 cc. water produced an immediate crystalline precipitate (7·74 g.). Recrystallisation of this platinum salt from water yielded 5·9 g. *ammonium chloroplatinate.*

Analysis. 0·2793 g. gave 0·1228 g. Pt.

Pt: found 43·96 %; calc. for $(NH_4)_2PtCl_6$, 43·91 %.

The solution from which the insoluble platinum salt had been removed was evaporated to about 50 cc. when the addition of 3 g. platinum chloride dissolved in a little water produced a further crop of 2·5 g. of insoluble platinum salt. After platinum had been removed from the filtrate by means of H_2S the solution was made alkaline and steam-distilled. Half of the distillate, which smelt strongly of pyrrolidine, was titrated with *N* HCl, requiring 17·3 cc. for neutralisation (the other half was worked up in a different manner), and then concentrated to small bulk. The precipitate produced by the addition of gold chloride weighed 1·45 g. This was recrystallised from 1 % HCl, yielding stout prismatic needles. After three recrystallisations the melting-point remained constant (199–205° with decomposition), 0·292 g. being obtained. On analysis it was found to contain 47·17 % Au: pyrrolidine chloroaurate (M.P. 205–6°), 47·94 % Au: *N*-methylpyrrolidine chloroaurate (M.P. 218°), 46·36 % Au.

Since the analysis suggested the presence of a small amount of *N*-methylpyrrolidine (?) the chloroaurate was converted into the chloroplatinate, which crystallised in needles on adding alcohol to its aqueous solution.

From water it crystallised in orange-red columns and from aqueous alcohol in needles (M.P. 208° with decomposition). It was identified as *pyrrolidine chloroplatinate.*

Analysis. 0·1174 g. gave 0·0413 g. Pt.

Pt: found, 35·18 %; calc. for pyrrolidine chloroplatinate, 35·32 %; for methylpyrrolidine chloroplatinate, 33·62 %.

(*b*) *Attempted identification of* N-*methylpyrrolidine.*

The properties of the corresponding salts of pyrrolidine and *N*-methylpyrrolidine are, as is to be expected, very similar, the only pair displaying marked difference in melting-points being the picrates:

pyrrolidine picrate, 112°; *N*-methylpyrrolidine picrate, 218°.

In the following experiment, therefore, an attempt was made to separate the two bases by recrystallisation of the picrates.

0·52 g. spermine hydrochloride was decomposed and steam-distilled as in (*a*). The distillate, after neutralisation, was evaporated to 20 cc. and the pyrrolidine bases were precipitated by the addition of Dragendorff's reagent (potassium bismuth iodide solution). The precipitate was suspended in 8 %

NaOH solution and steam-distilled. The distillate was neutralised with picric acid and evaporated to dryness. The dry residue was recrystallised from absolute alcohol, thin leaflets being obtained. After three recrystallisations the amount remaining sufficed only for a melting-point determination. It softened at 200°, melting to an oil at 208–214°.

It was, therefore, probably a still slightly impure specimen of *N*-methylpyrrolidine picrate.

II. Degradation of decamethylspermine sulphide.

8 g. spermine hydrochloride were methylated and the mercurichloride of the quaternary ammonium base was isolated as described in the second paper of this series [Dudley and Rosenheim, 1925]. The mercurichloride was suspended in 3 l. of hot water and decomposed with H_2S. After evaporating the filtrate to small bulk it was treated with excess of silver oxide. After filtration the solution of the free base was saturated with H_2S and then concentrated *in vacuo* (water-bath at 45–50°). The solution of the sulphide was run into a small Claisen flask immersed in a bath at 130–135°. When the distillation of water had slackened, the temperature of the bath was raised to 150°. A watery basic distillate (fraction A) boiling at about 100° was collected, the temperature of the bath being slowly raised to 180°. The residue in the flask was then distilled *in vacuo*. A small fraction boiling below 100° was collected separately and the main high-boiling fraction (B) distilled between 100° and 120° at 15–20 mm. pressure while the temperature of the bath was raised gradually to 220°.

Identification of tetramethyltrimethylenediamine.

Fraction A required 12 cc. 10 % HCl for neutralisation, and contained a small quantity of an oil which had a garlic-like odour. This was shaken out with ether after a slight excess of HCl had been added. The ethereal solution gave the heavy white precipitate, characteristic of alkyl sulphides, on addition of alcoholic $HgCl_2$ solution.

From the solution of the hydrochloride, sodium picrate precipitated a picrate which, after recrystallisation from water, melted at 198–202° (yellow oil), but was not quite homogeneous: yield 4·9 g. It was converted into the hydrochloride, the solution of which was evaporated to dryness. A small quantity of this salt in alkaline solution was treated with benzenesulphonyl chloride and the original picrate was recovered after this treatment, proving that the base was tertiary as anticipated. Judged by the properties of the recovered picrate, the base appeared to have undergone a slight purification during this process, to which, accordingly, the main bulk of the hydrochloride was subjected. It was dissolved in 6 % NaOH and shaken with 4 cc. benzenesulphonyl chloride, and, after the latter had disappeared, the still alkaline liquid was steam-distilled. Picric acid was added to the distillate and 4·4 g.

picrate were recovered which appeared to be quite homogeneous, crystallising in long flat needles; M.P. 202–204° (yellow oil).

The properties of the picrate, chloroaurate and chloroplatinate, together with the analyses of these salts, are tabulated below (Tables I and II).

The apparently low boiling-point of the tertiary base, the physical properties and analyses of its picrate and chloroaurate all suggested, on reference to the literature, its identity with ethyldimethylamine. A specimen of this base was therefore prepared by Eschweiler's method [1905] and the assumption disproved by mixed melting-point determinations:

	Chloroaurates M.P.	Picrates M.P.
Tertiary base	216° (decomp.)	202–4° (to oil)
Ethyldimethylamine	218° ,,	202–4° ,,
Mixed sample	196–8° ,,	170° ,,

The molecular weight of the picrate was then determined by Rast's method [1923; see also Ssadikow and Winogradow, 1924].

		Δ	M.W.
(i)	5 mg. picrate dissolved in 100 mg. camphor	3·3	606
(ii)	,, ,, 50 mg. ,,	6·5	615
	Calculated for $C_9H_{20}N_2(C_6H_3O_7N_3)_2$ ...	—	602

This finding made it clear that the tertiary base had a molecular weight approximately twice that of ethyldimethylamine and, therefore, contained two tertiary nitrogen atoms. Since the $>N.[CH_2]_4.N<$ group had already been detected in spermine it was at first thought that the tertiary base might be tetramethyltetramethylenediamine. The fact that the fraction containing the tertiary base had been collected at 100°, whilst tetramethyltetramethylenediamine boils at 169° was easily explainable because water was present during this stage of the degradation and fraction A was in effect a steam-distillate.

Comparison of the data recorded for this base by Willstätter and Heubner [1907] with those found for the tertiary base revealed similarity between the picrates and chloroaurates but disagreement in the case of the chloroplatinates. The tertiary base was therefore methylated in the usual way and compared directly with the quaternary ammonium salt prepared by exhaustive methylation of a gram of synthetic putrescine hydrochloride [Dudley and Thorpe, 1925] which was immediately available. Again discrepancies were encountered which excluded the identity of the tertiary base with tetramethyltetramethylenediamine. The small amount of synthetic putrescine in hand precluded the direct comparison of the two tertiary bases themselves.

The analyses of the various salts of the tertiary base from decamethylspermine and its quaternary ammonium derivative are consistently rather high for tetramethyltetramethylenediamine and agree well with those required for the corresponding derivatives of tetramethyltrimethylenediamine.

The latter base was therefore synthesised according to the directions of Clarke [1913], and a portion was methylated.

Direct comparison then proved conclusively the identity of the tertiary base from decamethylspermine with tetramethyltrimethylenediamine.

The evidence is collected in the following three tables.

Table I. *Analyses.*

Salt analysed	Found for tertiary base from decamethylspermine	Required for tetramethyltrimethylenediamine ($C_7H_{18}N_2$)	Required for tetramethyltetramethylenediamine ($C_8H_{20}N_2$)
Picrate:			
(0·0809 g. gave 0·1484 g. nitron picrate)	77·65	77·90	76·09 %
Chloroaurate:			
(0·1575 g. gave 0·0757 g. Au)	48·07	48·65	47·82 %
(0·2162 g. gave 0·1046 g. Au)	48·38		
Chloroplatinate:			
(0·2253 g. gave 0·0805 g. Pt)	35·73	36·11	35·20 %
Quaternary ammonium bases			
Chloroaurate:			
(0·1681 g. gave 0·0780 g. Au)	46·40	47·02	46·25 %
Chloroplatinate:			
(0·1917 g. lost 0·0056 g. H_2O at 103°)	2·92	3·07 (for 1 H_2O)	Contains no H_2O
(0·1861 g. (dry) gave 0·0638 g. Pt)	34·29	34·33	33·51 %

Table II. *Comparison of tertiary bases.*

	Tetramethyltrimethylenediamine (synthetic)	Tertiary base from decamethylspermine	Tetramethyltetramethylenediamine (W. and H.'s data)
Picrates	Flat thin needles M.P. 206–7° (yellow oil)	Flat thin needles M.P. 202–4° (yellow oil)	Long thin needles M.P. 198°
	mixed M.P. 202–4°		
Chloroaurates	Difficultly sol. in cold water	Difficultly sol. in cold water	Difficultly sol. in cold water
	Thick prisms and thin plates	Thick prisms and thin plates	Golden prisms
	Decomp. 214–5°	Decomp. 211–4°	Decomp. 206–7°
	mixed M.P. 212–4°		
Chloroplatinates	Very sol. in cold water	Very sol. in cold water	Difficultly sol. in cold water
	Prismatic columns	Prismatic columns	
	No H_2O of crystn.	No H_2O of crystn.	2 H_2O of crystn.
	Decomp. 240–4°	Decomp. 248–50°	Decomp. 234°; 238–9° (Clarke)

Identification of hexamethylspermine.

On addition of water to fraction B a small quantity of oil with a strong garlic-like odour separated and was removed by shaking with ether after the solution, which required 6·5 cc. 10 % HCl for neutralisation, had been made slightly acid to Congo red. After boiling the solution with charcoal and filtering, excess of 20 % gold chloride solution was added. The chloroaurate obtained was recrystallised from 2·5 % HCl; a smell of chlorine (?) was noticed and there was deposition of a small amount of metallic gold. The salt crystallised in balls of minute needles. Yield 5·6 g. Recrystallisation of this salt failing to give a pure compound (unsaturated substances were present), it was dis-

Table III. *Comparison of quaternary ammonium bases.*

Hexamethyltrimethylene-diammonium hydroxide (synthetic)	Methylated tertiary base from decamethylspermine	Hexamethyltetramethylene-diammonium hydroxide (Synthetic)	Hexamethyltetramethylene-diammonium hydroxide (W. and H.'s data)
Picrates			
Flat, square-ended plates	Flat, square-ended plates	Long, flat plates	Aggregates of prismatic needles
Decomp. 284–6°	Decomp. 282–6°	Not decompd. at 294°	Decomp. 285°
mixed M.P. 282–6°	mixed M.P. 282–6°		
Chloroaurates			
Very insol. in water Dull prismatic needles Decomp. 280–2°	Very insol. in water Dull prismatic needles Decomp. 286–8°	Very insol. in water Short thick prisms Decomp. 300–2°	Very insol. in water Aggregates of prisms Decomp. 304–9°
mixed M.P. 276–80°	mixed M.P. 294–8°		
Chloroplatinates			
Thin lustrous plates* (orange-yellow prismatic needles) Not sol. in 7 parts boiling water 1 H_2O of crystn. Decomp. 282–6°	Orange-yellow* prismatic needles Not sol. in 7 parts boiling water 1 H_2O of crystn. Decomp. 280–4°	Orange-yellow prismatic needles Sol. in 7 parts boiling water No H_2O of crystn. Decomp. 284–6°	Orange-yellow prismatic needles Sol. in 7 parts boiling water — Decomp. 279°
mixed M.P. 281–5°	mixed M.P. 276–80°		
Mercurichlorides			
Prismatic needles Decomp. 268–70°	Prismatic needles Decomp. 266–70°	Prismatic needles Decomp. 266–70°	— —
mixed M.P. 264–8°	mixed M.P. 244–8°		

* This salt is dimorphic.

solved in dilute HCl and decomposed by means of H_2S. The slightly hygroscopic hydrochloride obtained was dissolved in a small amount of water, alcohol was added until a permanent turbidity was produced and the solution on standing at 2° for 2 days deposited crystals. These were dissolved in boiling methyl alcohol, the solution was filtered and ether was added until a faint turbidity was produced. After standing at 2° the crystalline *hydrochloride*, no longer hygroscopic, was filtered off, 0·44 g. being obtained: M.P. (with decomposition), 273–276°.

Analysis. 0·1146 g. (dried at 100°) gave 0·1868 g. CO_2 and 0·0943 g. H_2O. 0·1310 g. (dried at 100°) gave 0·1723 g. AgCl.

	C	H	Cl
Found	44·45 %	9·14 %	32·53 %
Calculated for hexamethylspermine ($C_{16}H_{38}N_4$. 4 HCl)	44·44	9·72	32·87

From the pure hydrochloride the *chloroaurate* was prepared. It was recrystallised from 5 % HCl yielding pale yellow rosettes of needles, M.P. (decomposition) 200–203°, which contained no water of crystallisation.

Analysis. 0·1782 g. gave 0·0850 g. Au.

Au: found, 47·70 %; calc. for $C_{16}H_{38}N_4.4HAuCl_4$, 47·88 %.

The identification was clinched by methylating the remainder of the hydrochloride in the usual manner and comparing the resulting quaternary ammonium base with that from spermine (decamethylspermine).

The *picrate* and *chloroaurate* were prepared and melted identically with the corresponding salts of decamethylspermine [Dudley and Rosenheim, 1925], mixed melting-point determinations showing no depression.

Analyses. 0·0872 g. picrate gave 0·1505 g. nitron picrate.

0·1755 g. chloroaurate gave 0·0810 g. Au.

	Picric acid	Au
Found	73·05 %	46·16 %
Calculated for salts of $C_{20}H_{50}N_4$	72·82	46·30

III. Oxidation of, and action of nitrous acid on spermine.

Oxidation. Spermine is readily oxidised by a variety of reagents, but since the study of the products formed did not contribute directly to the elucidation of the structure of the base, the experiments will not be described in detail. Vigorous oxidation with $KMnO_4$ and with H_2O_2 yielded a small quantity of an acid, probably succinic, derived doubtless from the tetramethylene group in spermine.

Milder oxidation gives a volatile base with which the characteristic odour of semen is associated. This substance is constantly formed under conditions in which oxidative reactions may occur. If, for instance, an aqueous suspension of spermine chloroaurate is heated on the water-bath a copious precipitate of metallic gold is produced while the spermine is simultaneously partially oxidised; the action of magnesium on spermine chloroaurate, described by Schreiner [1878] involves, in all probability, a similar oxidation. Further, when a solution of spermine is warmed with silver oxide, the volatile base is produced and at the same time metallic silver is deposited as a mirror. Since this mirror is not produced in the presence of "dimedon" it is possible that one of the first substances formed in the reaction is an aldehyde.

The base is volatile in steam and forms a crystalline, readily oxidisable hydrochloride and a chloroaurate (M.P. 204–206° (with decomposition): Au = 48·45 %). Apparently Wrede [1926] obtained the same base from the products formed by oxidising spermine with metallic copper and air, but did not succeed in identifying it.

In the light of the now established constitution of spermine it is suggested that the new base may have the structure of a *N*-γ-aminopropylpyrroline. The data so far recorded by Wrede are in agreement with this assumption which can easily be tested by synthesis.

Action of nitrous acid. The volatile base mentioned above is also formed by the action of HNO_2 on spermine, together with a substance giving a nitroso-reaction. In view of the complexity of the reaction it is not surprising that

Van Slyke determinations give inconclusive results. Varying proportions of gas, measured as nitrogen, are evolved according to the length of time the reaction is allowed to proceed. In a series of determinations quantities of gas were measured corresponding to 65 % of the total N after 30 minutes, 76 % after 1 hour and 85 % after 2 hours. Wrede [1926] obtained 83 % of the theoretical quantity after 1 hour under the conditions of his experiments and assumed from this result that spermine contains three NH_2-groups and, since it forms tetraacyl derivatives, one NH-group. These assumptions are disproved by the combined results of methylation and benzoylation reported in the previous papers of this series and by the synthesis described in the present communication.

IV. Synthesis of spermine

α.δ-Bis[γ'-phenoxypropylamino]-butane hydrobromide.

6·3 g. tetramethylenediamine[1], distilled over sodium, and 60 g. α-phenoxy-γ-bromo-propane, prepared according to v. Braun and Beschke's directions [1906], were dissolved in 180 cc. absolute alcohol and heated on a water-bath under a reflux condenser. Within 25 minutes crystalline material began to separate and after 4 hours' heating this was filtered off, 16·1 g. being obtained. This consisted of a mixture of the desired diphenoxy-compound together with some α-(γ'-phenoxypropylamino)-δ-amino-butane hydrobromide, which was found also in considerable quantity in the alcoholic filtrate.

On recrystallisation from 260 cc. water the diphenoxy-compound was obtained in a pure condition; yield 9·2 g.; long thin plates, M.P. 292° (dark-red liquid).

Analysis. 0·1042 g. gave 0·0750 g. AgBr.

Br: found, 30·64 %; calc. for $C_{22}H_{34}O_2N_2Br_2$; 30·88 %.

From this hydrobromide the *picrate* was prepared, crystallising from 90 % alcohol in needles, M.P. 178–180° (brown oil).

α.δ-Bis[γ'-bromopropylamino]-butane hydrobromide.

5 g. of the above phenoxy-compound were heated in a sealed tube with 25 cc. HBr solution (Sp.G. 1·7) at 100° for 18 hours.

On shaking the contents of the tube with ether to remove the phenol formed in the reaction, a crystalline substance separated from the aqueous layer (yield 4·55 g.). After recrystallisation from 250 cc. 96 % alcohol 3·24 g. were obtained.

The substance, which was found to be the desired dibromo-compound, forms glistening broad plates and melts with effervescence at 274°.

[1] The putrescine hydrochloride used in this synthesis was prepared by Dr J. Marshall, of Boots' Pure Drug Co., Nottingham, who very kindly presented us with a very pure specimen of this salt, enabling us to carry out the synthesis on a small scale. For this and the larger quantities with which he has subsequently supplied us, we wish to tender our best thanks.

Analysis. 0·1074 g. gave 0·1646 g. AgBr.

Br: found, 65·21 %; calc. for $C_{10}H_{24}N_2Br_4$, 65·03 %.

From the hydrobromide the *picrate* was prepared. After recrystallisation from 96 % alcohol it was obtained as prismatic crystals, M.P. 186–188° (brown oil).

α.δ-Bis[γ'-aminopropylamino]-butane phosphate.
(Synthetic spermine phosphate.)

4·55 g. of the above bromo-compound were heated in 1–2 g. lots with approximately 20 volumes of alcoholic ammonia (15–20 % by weight) in sealed tubes at 100° for 7 hours. The alcohol and excess of ammonia were then removed by distillation *in vacuo*, leaving a white crystalline residue[1]. This was dissolved in water and solid NaOH was added to give a 50 % solution which was then steam-distilled. From the distillate (1500 cc.) 1·3 g. insoluble phosphate was obtained by the method already described for the isolation of natural spermine phosphate [Dudley, M. C. Rosenheim and O. Rosenheim, 1924]. For analysis it was recrystallised from water and the *picrate*, *picrolonate*, *chloroaurate*, *chloroplatinate* and *benzoyl-derivative* were prepared from it. Comparison of these salts with those of spermine isolated from animal organs revealed complete identity, as judged by crystalline form, melting-points and mixed melting-point determinations.

Analyses.

Phosphate. 0·1825 g. lost 0·0396 g. H_2O at 105°.

H_2O found; 21·69 %.

Calc. for $C_{10}H_{26}N_4.2H_3PO_4$, $6H_2O$: H_2O, 21·34 %.

0·1429 g. dry salt gave NH_3 (Kjeldahl) = 14·39 cc. $N/10$ H_2SO_4.

0·2117 g. dry salt gave 0·1180 g. $Mg_2P_2O_7$.

Found: N, 14·10 %; P, 15·53 %.

Calc. for $C_{10}H_{26}N_4.2H_3PO_4$: N, 14·07 %; P, 15·56 %.

Picrate. 0.0497 g. gave 0·0962 g. nitron picrate.

Found: picric acid, 81·92 %.

Calc. for $C_{10}H_{26}N_4.4C_6H_3O_7N_3$: picric acid, 81·98 %.

Chloroplatinate. 0·1931 g. lost 0·0134 g. at 105° and the dry salt gave 0·0688 g. Pt.

Found: Pt, 38·28 %.

Calc. for $C_{10}H_{26}N_4.2H_2PtCl_6$: Pt, 38·17 %.

The proof of the structure of spermine afforded by this investigation raises several points of biochemical interest. This is the first instance of a base of the type $NH_2.R.NH.R'.NH.R.NH_2$ being found in nature. It is possible

[1] Spermine phosphate can be precipitated directly from an aqueous solution of this residue by means of ammonium phosphate, but it is contaminated with insoluble phosphates of secondary reaction products which are difficult to separate by recrystallisation.

that future work may disclose the presence of similar compounds in living tissues, and furnish a clue to the origin and function of this interesting base[1].

The soluble phosphate, already reported as accompanying spermine in the processes of isolation from animal tissues [Dudley, M. C. Rosenheim and O. Rosenheim, 1924] may prove to be a salt of a base of this type and the question is now receiving attention.

In connection with the extraordinary insolubility of spermine phosphate it is interesting to note that a base of similar structure, namely "triethylenetetramine"[2] $NH_2[CH_2]_2.NH.[CH_2]_2.NH.[CH_2]_2.NH_2$ does not form an unusually insoluble phosphate.

The presence in spermine of the trimethylenediamino-group is of considerable interest. The occurrence of trimethylenediamine, or its derivatives, in nature has not hitherto been established, although it has been reported.

Brieger [1887] isolated from cultures of *V. cholerae*, besides putrescine and cadaverine, a base whose elementary composition agreed with that of trimethylenediamine, but the substance has never been re-investigated.

Njegovan [1912], working on bases from plant phosphatides, obtained a gold salt which he considered might be that of completely methylated trimethylenediamine. The evidence, however, is not in the least convincing and has, indeed, been vigorously challenged by Trier [1912].

In spermine we have, indisputably, a derivative of trimethylenediamine, a fact which renders it more probable that the base itself may be encountered among the products of biological activity.

Summary.

1. Methylation and benzoylation of spermine proved that the base contains two NH_2-groups and two NH-groups.

2. The presence of the chains N—C—C—C—C—N and N—C—C—C—N was inferred from the identification of pyrrolidine among the products of destructive distillation of spermine hydrochloride, and of tetramethyltrimethylenediamine obtained by the degradation of decamethylspermine.

3. The following structural formula for spermine, based on these observations, was proved to be correct by synthesis:

$$NH_2.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.CH_2.NH.CH_2.CH_2.CH_2.NH_2$$

($\alpha.\delta$-bis[γ'-aminopropylamino]-butane).

[1] The pharmacological action of spermine has been investigated by Prof. Gibbs in this Institute and the results will be published shortly.

[2] We are indebted to Dr T. A. Henry for a specimen of this base.

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CXXXIX. STUDIES ON XANTHINE OXIDASE.

VIII. THE OXIDATION-REDUCTION POTENTIAL OF THE OXIDASE SYSTEM.

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I. Introduction.

Since the publication of the valuable work of Biilmann and Lund [1921] on the oxidation-reduction potential of quinone derivatives and other organic substances, much attention has been given to the potentiometric study of the chemical affinity of oxidation-reduction reactions. In biological research, however, it is still in its infancy and its importance is not yet fully appreciated. The work described in the present communication was undertaken with the object of showing that the potentiometric method may be a valuable means for the study of biological oxidation systems.

It was decided to investigate a typical oxidation-reduction enzyme with a view to determining how far the potentiometric behaviour of such a system would resemble that of the (non-biological) oxidation-reduction systems so much studied from this point of view.

As a typical oxidation-reduction enzyme xanthine oxidase was chosen, owing to the ease of preparation of the enzyme in a purified form. Our present knowledge of this enzyme is due chiefly to the work done by Morgan, Stewart and Hopkins [1922] on its existence in milk, followed by the extensive studies of Dixon and Thurlow on the physico-chemical aspects of its action.

In presence of this enzyme hypoxanthine and xanthine are oxidised to uric acid by hydrogen acceptors such as molecular oxygen or methylene blue, etc. The reaction may be pictured as follows:

$$\underset{\text{Enzyme}}{E} + \underset{\text{Xanthine}}{X} + H_2O = EXOH_2$$

$$EXOH_2 + \underset{\substack{\text{Hydrogen}\\\text{acceptor}}}{A} = E + \underset{\text{Uric acid}}{XO} + AH_2$$

in accordance with Wieland's theory of oxidation. The enzyme and its substrate first combine to form a complex of the type represented. The two hydrogen atoms of this complex are "active" and readily transferred to a hydrogen acceptor, as in the second equation. Many reducible substances can act as hydrogen acceptors in this system [Dixon, 1926], but those which have been most studied are molecular oxygen and methylene blue. In the first case the oxygen uptake can be measured by means of Barcroft's apparatus, and in the second case the reaction can be followed by the disappearance of the blue colour, using Thunberg's vacuum tubes.

II. Experimental technique.

The reaction vessel used was one of about 15 cc. capacity. It was tightly fitted with a rubber stopper with five holes, holding tubes for the gas inlet and outlet, the electrode, burette and agar salt bridge. The electrode consisted of a gold plate of 1 cm.^{2}. Platinum proved less satisfactory, owing to its slowness in following the change of the potential as the reaction proceeded. As the other half-element a saturated calomel electrode was used, which was connected with the reaction vessel by means of the agar salt bridge. The whole apparatus was immersed in a water thermostat kept at 18°. For the measurement of the potential the Unipivot type of potentiometer of the Cambridge Instrument Company was used. The readings were accurate to 1 millivolt.

III. Does hypoxanthine plus xanthine oxidase give a reduction potential?

Clark [1925] carried out an experiment on the Schardinger enzyme in fresh milk, which acts upon aldehydes in exactly the same way as the xanthine oxidase does upon xanthine (the two enzymes may be identical). He states that in presence of milk aldehyde becomes activated in such a way as to give a high reduction potential, which is not given by the aldehyde alone or by aldehyde plus boiled milk. The author, however, has been entirely unable to repeat these results, in spite of repeated attempts to do so[1]. This was the case both with fresh milk or with the purified preparation of the enzyme, and whether formaldehyde, acetaldehyde or hypoxanthine was used as hydrogen donator. Some experimental details are as follows.

5 cc. of a fresh milk, which had been tested and found to be very active with the methylene blue technique, were pipetted into the reaction vessel, and nitrogen gas, which had passed through two wash-bottles containing strong pyrogallol solution, bubbled through it. When the potential showed a steady value, 1 cc. of 1 % acetaldehyde, which was previously neutralised with sodium hydroxide, was introduced through the burette and the change of the potential was followed. Similar experiments with hypoxanthine were repeated, using 0·5 g. of the enzyme preparation obtained by the method of Dixon and Thurlow [1924, 1] or 0·002 g. of the enzyme purified by the method of Dixon and the author published elsewhere in this *Journal* [1926]. Each enzyme preparation was dissolved in 5 cc. of phosphate buffer solution ($p_H = 7{\cdot}6$) and 1 cc. of hypoxanthine solution, containing 0·2 mg., was added instead of the acetaldehyde. The results of these measurements are illustrated in Fig. 1, where E_h is plotted as ordinate and time as abscissa.

As will be seen from Fig. 1, the fresh milk and the caseinogen preparation gave only a slight change in potential on the addition of acetaldehyde and hypoxanthine respectively, while the purified preparation of the

[1] Dr M. Dixon has also been unable to repeat Clark's results.

same activity as 5 cc. of the fresh milk gave a still smaller change with these hydrogen donators. Possibly some reducible substance exists in the fresh milk which gives this slight potential when reduced.

In no case has any potential change of the order given by Clark been observed with fresh milk or with the enzyme preparations[1]. Occasionally, however, curves similar to Clark's have been obtained when a not very fresh milk was used, but these potential changes were obviously due to bacteria, and were *not* dependent on the presence of aldehyde. It is significant that the final potential reading in these experiments, which was the same as that obtained by Clark after inoculating the milk with bacteria, was identical with the final value given by Clark for the Schardinger system; this fact suggests that the effects he observed may possibly have been due to bacteria.

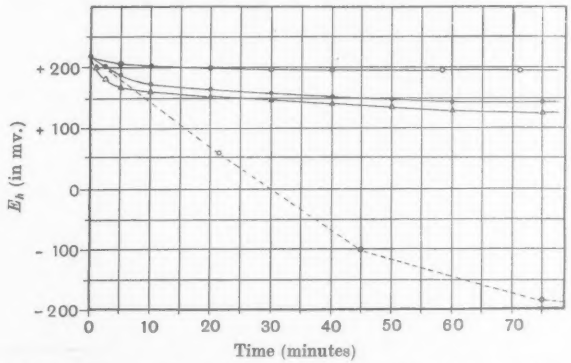

Fig. 1. The reduction potential of xanthine oxidase + hypoxanthine.

–○——○–	Purified oxidase preparation + hypoxanthine.	p_H 7·6. 14°
–●——●–	Caseinogen preparation + hypoxanthine.	p_H 7·6. 14°
–△——△–	Fresh milk + aldehyde.	p_H 7·0. 14°
··○ ····○	Clark: Fresh milk + aldehyde	p_H 7·0. 37°

It is, moreover, clear that the potentials observed by Clark cannot represent the true reducing potential of the enzyme system; for it follows from a consideration of the curve given by him that the apparent reduction potential only reaches a value theoretically sufficient to begin to reduce methylene blue about 30 minutes after addition of the aldehyde, whereas actually under similar conditions the reduction begins at once and may in fact be complete in under 1 minute.

IV. The oxidation-reduction potential in presence of a hydrogen acceptor.

This potentiometrically inactive system (xanthine oxidase + hypoxanthine or aldehyde), however, exerts its full reducing power when it comes in contact with reducible substances. This is the extremely interesting and characteristic feature of biological oxidation-reduction systems, namely that, while giving

[1] Experiments carried out at 37° gave results similar to those at 14°.

no reduction potential themselves, they are yet able to bring about the reduction of a large number of hydrogen acceptors. The following experiments show the effect on the potential of the presence of various hydrogen acceptors.

(a) The potential in presence of oxygen.

2 mg. of the purified enzyme dissolved in 6 cc. of the phosphate buffer ($p_H = 7{\cdot}6$) were placed in the reaction vessel and air bubbled through. When the potential had attained a steady value, 1 cc. of hypoxanthine solution containing 0·2 mg. was introduced from the burette and the change of the potential was followed. The results are shown in the following diagram (Fig. 2).

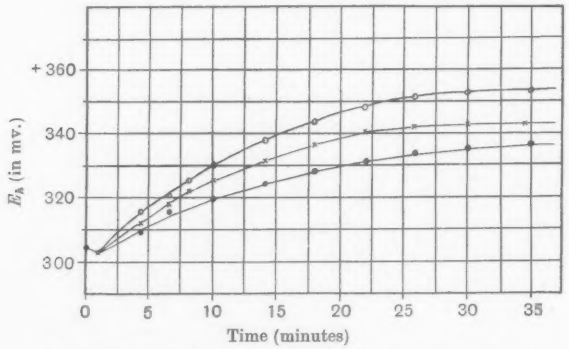

Fig. 2. The oxidation-reduction potential in presence of air.
–○——○– 4 mg. oxidase + 0·5 mg. hypoxanthine
–×——×– 2 mg. ,, + 0·5 mg. ,,
–●——●– 2 mg. ,, + 0·2 mg. ,,

As shown in the above figure, the potential shifts in the direction of higher oxidation potential on the addition of hypoxanthine when oxygen is present. This effect might be expected from the results of Thurlow [1925], who showed that in this system hydrogen peroxide is formed, which will give an oxidation potential.

It will be interesting to compare this with the following results, which were obtained by adding pure dilute hydrogen peroxide solution of known strength to a solution of 2 mg. of enzyme in 5 cc. of phosphate buffer. By this means we may obtain some idea of the amount of hydrogen peroxide formed.

The results obtained are given in Fig. 3, where the observed potentials are plotted against the logarithm of the volume (cc.) of hydrogen peroxide added. 1 cc. of the solution used contained 0·0118 mg. H_2O_2.

The curve is obviously a straight line, and can be represented by the equation

$$E_h = K + a.\log C,$$

where E_h is the observed potential reduced to the normal hydrogen scale, C is the concentration of hydrogen peroxide, and K and a are constants,

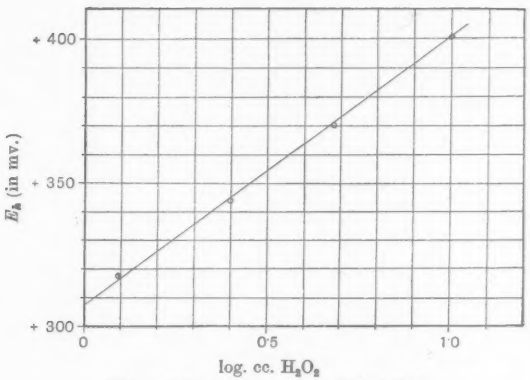

Fig. 3. The oxidation potential of H_2O_2.

which in this case have the values of 308 and 93 mv. respectively. From this equation we can calculate, from the values of E_h observed in the experiments of Fig. 2, the amount of hydrogen peroxide produced in that experiment, as in the following table.

Table I. *The amount of hydrogen peroxide produced.*

Xanthine oxidase mg.	Hypoxanthine mg.	E_h mv.	Corresponding amount of H_2O_2 mg.	Theoretical amount mg.
2	0·2	+337	0·0236	0·0625
2	0·5	+343	0·0279	0·125
4	0·5	+354	0·0365	0·125

The theoretical value is calculated on the assumption that one molecule of hypoxanthine gives rise to two molecules of hydrogen peroxide. The actual amount of hydrogen peroxide found is, however, smaller than this, owing to the fact that the peroxide molecules may react further with activated hydrogen atoms from the hypoxanthine, as follows: $H_2O_2 + 2H = 2H_2O$. This reaction was shown to occur by Thurlow.

(*b*) *The potential in presence of methylene blue.*

As already mentioned, xanthine oxidase plus hypoxanthine does not give any reduction potential to account for the reduction of methylene blue; it is a well-known fact, however, that the dye is rapidly reduced. Now the oxidised and reduced forms of the dye, as shown in detail by Clark [1925], give a reversible oxidation-reduction potential, which can be calculated (for a given p_H) by Peters' equation:

$$E_h = E_k - \frac{RT}{nF} \log \frac{[\text{Red}]}{[\text{Ox}]},$$

where E_h R, T, n and F have their usual significance, and E_k is a constant characteristic of this particular oxidation-reduction equilibrium. [Red] and [Ox] are the concentration of the reduced and oxidised forms of the dye respectively. By means of this equation the percentage reduction of the methylene blue can be calculated from the potential value.

Since the oxidase-hypoxanthine system by itself gives no potential, as has been shown in the previous section, it may be expected that the potential observed anaerobically in presence of methylene blue will simply be due to the latter, so that the percentage reduction of the dye at any instant may be determined by measuring the potential given by the system.

In order to see whether this was actually the case the following experiment was carried out, in which the methylene blue was reduced step by step by adding successively small known amounts of hypoxanthine. After each addition the potential was determined after sufficient time had elapsed to allow the reaction between the hypoxanthine and the methylene blue to be completed and the potential to become steady. As one molecule of hypoxanthine reduces two molecules of methylene blue, as shown by Morgan, Stewart and Hopkins [1922], the percentage reduction of the methylene blue could be calculated from the amount of hypoxanthine added.

5 cc. of phosphate buffer solution ($p_H = 7{\cdot}6$) and 0·5 cc. of methylene blue (1 : 5000) were introduced into the reaction vessel, and nitrogen gas was bubbled through. After the potential showed a steady value, which was usually attained in a few minutes, a solution of 2 cc. of enzyme solution (4 mg.) + 2 cc. hypoxanthine solution (0·4 mg.) + 4 cc. distilled water was delivered from the burette 0·1 cc. at a time. At the various stages of reduction the potential was read when it became constant.

For comparison a similar experiment was carried out under the same conditions using standard titanous chloride solution to reduce the methylene blue instead of the hypoxanthine.

The two series of results are compared in Fig. 4 with the curve calculated from Peters' equation above.

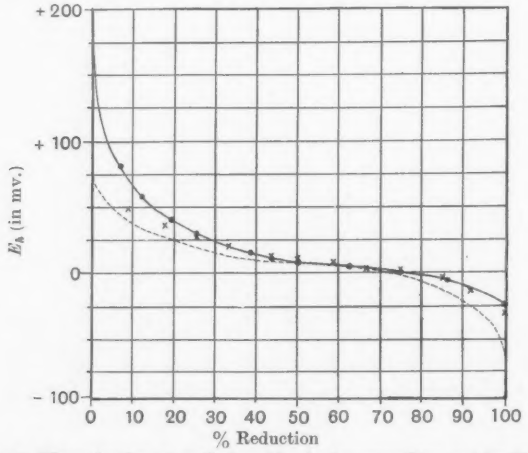

Fig. 4. The reduction of methylene blue by hypoxanthine and by $TiCl_3$.
–●——●– Xanthine oxidase + hypoxanthine. –×——×– $TiCl_3$. - - - - - - Theoretical.

The good agreement of the curves shows that xanthine oxidase plus hypoxanthine behaves in a precisely similar way to the inorganic reducing agent. The slight divergence between the experimental and theoretical curves is probably due to traces of oxygen in the nitrogen gas and in the solutions. These would tend to make the actual percentage reduction values lower than the calculated, and would have the effect of displacing the experimental curves to the right.

Besides methylene blue the oxidation-reduction indicators described by Clark and his co-workers [1923, 1, 2, 3, 4] can be reduced by the xanthine oxidase plus hypoxanthine, as shown by Dixon [1926]. The author has studied the potential of this system in presence of the following dyes. In all cases excess of hypoxanthine was used, and ample time was allowed for the reaction to proceed to completion. The final r_H values have been calculated in the usual way from the potential measurements according to the equation

$$r_H = 2\left(\frac{F.E_h}{RT} + p_H\right),$$

and compared with those from Clark's data for 99 % reduction.

Table II.

Dye	Final E_h mv.	r_H Found	r_H Clark
o-Chlorophenol-indophenol	+128	19·9	19·4
p-Cresol-indophenol	+107	19·1	18·5
1-Naphthol-2-sulphonic acid-indophenol	+ 12	15·6	15·6
Methylene blue	− 32	14·0	13·6

Though the final r_H is a little higher than that found by Clark in most cases, this may be due to traces of oxygen. It appears from these results that the final observed potential depends simply upon the dye, being the potential given by the reduced form in each case.

It will be clear from the results which have been given that the potentiometric method gives us a useful means of following the course of the reduction of methylene blue by the oxidase system. In Fig. 5 is given a typical series of potential-time curves illustrating this reaction. The experiments were carried out anaerobically as before.

It will be seen that the potential at first follows the S-shaped curve of Fig. 4, indicating a constant-velocity reduction; the velocity then falls off quite suddenly at the end of the reaction, giving a sharp break in the curve at a point corresponding to the disappearance of the colour. This is in agreement with the observations of Dixon and Thurlow [1924, 2], who found by means of a colorimetric method that the reduction proceeded at a constant rate until the reaction was practically complete, the velocity only falling off just at the end. It will be noticed that the reaction velocity is proportional to the enzyme concentration, as Dixon and Thurlow state.

From the fact that the sharp break in the curves corresponds with the instant at which reduction is complete it follows that the potentiometric

method can be used for the exact determination of the reduction-time, this being the interval between the addition of the hypoxanthine and the occurrence of the break. As a matter of fact, when the activity of the enzyme to be tested is strong, there is no advantage in this method over the widely used vacuum-tube method, except that the personal error in judging the last nuance is eliminated. When the enzyme solution is weak, however, and half an hour or more is required for complete reduction, this method may be valuable, for the initial change of E_h is a function of the enzyme concentration. The details of experiments on this point will be published later.

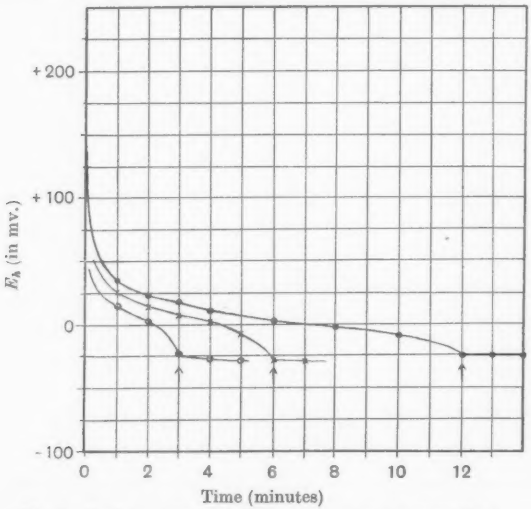

Fig. 5. E_h-time curve with different enzyme concentrations.
–●——●– 1 mg. enzyme –×——×– 2 mg. enzyme
–○——○– 4 mg. enzyme
Reaction system: 5 cc. phosphate buffer (p_H 7·6)
1 cc. enzyme solution, containing 1, 2 or 4 mg.
0·5 cc. methylene blue (1:5000)
1 cc. hypoxanthine, containing 0·2 mg.
The arrow indicates the point where colour disappeared.

Another application of the method is the qualitative and quantitative test as to whether a potentiometrically active substance is reduced by the enzyme or not, especially when the reduction of this substance is not accompanied by a colour change. For instance, the reduction of alloxan to alloxantin can be followed by this method.

SUMMARY.

1. Hypoxanthine and aldehyde do not give reduction potentials when activated by xanthine oxidase.

2. If methylene blue be added to this system a reduction potential is given; this is, however, merely the potential of the dye itself.

3. The course of the reduction of methylene blue by this system, and the reduction-time, can be determined by potential measurements.

4. In the presence of oxygen xanthine oxidase plus hypoxanthine give a potential. This is due to the formation of hydrogen peroxide as shown by Thurlow. The amount of hydrogen peroxide formed has been determined by potential measurements.

5. The oxidation-reduction indicators of Clark are reduced by xanthine oxidase and hypoxanthine, and the reduced forms then give their respective reduction potentials.

The author is greatly indebted to Sir F. G. Hopkins for his constant interest and encouragement, and also to Dr Dixon for his kind advice.

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CXL. ON THE FURTHER PURIFICATION OF THE XANTHINE OXIDASE.

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SINCE the discovery of the xanthine oxidase of milk by Morgan, Stewart and Hopkins [1922] two main attempts have been made to purify this enzyme further; namely, by Dixon and Thurlow [1924, 1], and later by Sbarsky and Michlin [1925].

Dixon and Thurlow obtained an active and stable oxidase preparation by precipitating the enzyme, together with the caseinogen and fat, by half-saturation of the milk with ammonium sulphate. The fat was removed from the dried precipitate by thorough extraction with ether. The resulting preparation is soluble, contains the whole amount of the enzyme, and is free from other milk enzymes such as peroxidase and catalase. Since the caseinogen content of milk is about 3 % it will be clear that this caseinogen preparation is about thirty times as concentrated as milk, *i.e.* 1 g. of the solid preparation contains as much enzyme as 30 g. of milk[1].

Sbarsky and Michlin's method of preparation is essentially similar. They use acetone instead of ammonium sulphate to precipitate the protein, and light petroleum instead of ether to extract the fat. The protein is, however, precipitated in an insoluble form by the acetone, and it is necessary to digest the preparation with dilute acid in order to get the enzyme into solution. The

[1] With regard to the activity of this preparation Sbarsky and Michlin state: "(Dixon and Thurlow) were able to salt out the caseinogen together with a portion of the enzyme. The enzyme content of the precipitate was however so small that they were obliged to use not less than 1·5 g. of the dry powder to bring about the reaction." There is here a complete misunderstanding of the statements of Dixon and Thurlow. They specifically stated that the whole of the enzyme was precipitated by the ammonium sulphate, and that the subsequent ether treatment did not diminish its activity, so that it is clear that the enzyme must have been concentrated to the extent given above. As a matter of fact, a few milligrams of the dry powder are quite sufficient to bring about the reaction actively. A point which seems to have contributed to the misunderstanding is the fact that in Dixon and Thurlow's studies on the dynamics of the enzyme the *strengths* of the enzyme solutions (as distinct from their *activities*) were omitted as having no significance. It may be stated here that the solutions used by Dixon and Thurlow were in general 1 or 2 % solutions, so that the average amount of preparation used in each experiment was about 50 mg., which gave very satisfactory velocities. The mention of "1·5 g." occurs only in connection with an adsorption experiment, in which it was desired to use a very large amount of the preparation.

preparation, moreover, contains the catalase and peroxidase of the milk in addition to the oxidase.

This method naturally gives a solid preparation of the same oxidase content as Dixon and Thurlow's preparation. Sbarsky and Michlin found, however, that in buttermilk the enzyme : protein ratio was several times higher than in milk; and by applying their method to buttermilk they obtained preparations about 240 times as active as the original milk from which the buttermilk was obtained.

The present communication gives the results of further work on the purification of this enzyme, together with a rapid and simple method for obtaining preparations of very greatly increased activity direct from milk.

The problem of further purification resolves itself into that of eliminating the protein without loss of enzyme. Up to the present attempts to purify Dixon and Thurlow's preparation further have not been successful. Precipitation and adsorption methods proved unsatisfactory, cataphoresis experiments at various p_H's showed that the oxidase always migrated with the caseinogen, and it cannot be dialysed away from the protein. We found, however, that, by making use of the fact discovered by Dixon and Thurlow that when the milk proteins are coagulated by the action of rennin a considerable part of the oxidase remains in the whey, it was possible to get rid of the greater part of the protein at the outset, and so obtain very much more active preparations. The procedure we adopted is as follows.

Rennin method of preparation.

The fresh milk, after its activity had been tested, was warmed to 35° and mixed with a small amount of rennin preparation ("Birks' Junket Powder"). The mixture was allowed to stand for 30 minutes, by which time it had set to a firm clot. This clot was then broken up by stirring, and the insoluble portion or curd was centrifuged off. The whey thus obtained was mixed with an equal volume of saturated ammonium sulphate solution, and allowed to stand. A small amount of precipitate formed, and quickly rose to the surface of the solution, where it formed a compact layer. The liquid was then siphoned off, and the precipitate transferred to a filter-paper, allowed to drain, and squeezed as dry as possible between filter-papers. It was then thoroughly extracted with ether to remove traces of fat. It was again squeezed between filter-papers, and finally dried in a vacuum desiccator. We shall refer to the resulting preparation as "the whey preparation."

It was found that when the milk clots the whole of the oxidase passes into the whey, and the curd, after washing with water, is quite inactive. Thus by this method the caseinogen and fat are got rid of without loss of enzyme.

The whole of the enzyme is precipitated by the half-saturation with ammonium sulphate.

The process of preparation should be carried out as quickly as possible, as the enzyme is much more easily destroyed by oxidation than in the case

of the caseinogen preparation. In particular, the drying must be carried out *in vacuo*. Allowing the preparation to dry in the air is sometimes sufficient to destroy the activity almost entirely, and it always considerably reduces it. The enzyme is perfectly stable in this form once the preparation is completely dry, and it can be kept in the vacuum desiccator for long periods without loss of activity.

The whey preparation is very readily soluble in water, giving a practically clear solution. The yield is about 10 g. from 1·5 l. of milk, and the activity was found by means of the methylene blue technique to be from 500 to 700 times that of the original milk.

It would appear from a comparison of the weights and activities given above that the final preparation contains more enzyme than the whole of the original milk. Since 1·5 l. of milk yield 10 g. of the whey preparation, this should be about 150 times as active as milk, whereas it is actually more than 500 times as active. The facts appear to suggest that the rennin sets free an additional quantity of xanthine oxidase from some inactive precursor in the milk. The rennin itself did not contain xanthine oxidase. Further work is necessary on this point.

The rennin method of preparation has been found most useful for general work, as it gives a means of obtaining, without difficulty, a soluble preparation of very high activity.

Methods for further purification.

(*a*) *Charcoal.* It was stated by Dixon and Thurlow that charcoal does not adsorb the oxidase from a solution of the caseinogen preparation. This observation was confirmed with the whey preparation. It was found, however, that in this case the charcoal did remove a considerable proportion of the protein. This suggested a method for further purification. The details are as follows.

1·5 g. of the whey preparation was dissolved in 150 cc. of distilled water. To the solution were added 3 g. of charcoal (Merck's medicinal), and it was then shaken for a few minutes and filtered through a Buchner funnel, using a Whatman filter-paper. A clear filtrate was obtained. After the enzyme activity and the relative protein content of this filtrate had been determined, a further quantity of charcoal was added and the above procedure repeated. This process was carried out repeatedly until the whole of the protein had been removed from the solution by adsorption. The results obtained are given in Table I.

For the estimation of the enzyme activity the following quantities were taken: 1 cc. of the filtrate + 1 cc. phosphate buffer (p_H 7·6) + 0·5 cc. methylene blue (1 : 5000) + 0·2 mg. hypoxanthine. The reduction took place at room temperature (15°), and by the activity we mean here the reciprocal of the reduction-time (in minutes) × 100 [cf. Dixon and Thurlow, 1924, 2].

The protein was estimated as follows: to 1 cc. of the filtrate were added

5 cc. of 2 % sulphosalicylic acid, and the resulting turbidity was compared in a nephelometer with that given by 1 cc. of the original solution treated similarly. Calling the protein content of the original solution 100 units, it is a simple matter to calculate the protein content of any filtrate in these units from the nephelometer readings. Sufficient time was always allowed for the turbidity to develop to its full extent before taking the readings.

Table I. *The adsorption of xanthine oxidase and the protein by charcoal.*

No. of adsorptions	Amount of charcoal used g.	Reduction time	Enzyme activity of filtrate	Relative protein content
0	0	1′ 30″	66·7	100
1	3	1′ 20″	75	67
2	3	1′ 15″	80	45·5
3	3	1′ 20″	75	30·7
4	2·4	1′ 35″ 1′ 40″	63	19·2
5	2	2′ 10″ 2′ 15″	46·2	12·8
6	2	5′ 0″	20	5·6
7	2	15′ 0″	6·5	2·1

It will be seen from the above results that at each stage the charcoal adsorbs 33 % of the protein in solution, except in the final stages, where the proportion is higher (about 50 % and 65 % in the sixth and seventh stages respectively). It will also be seen that the enzyme is not adsorbed by the charcoal until about 80 % of the protein has been removed, after which the enzyme is adsorbed together with the protein. The slight rise in activity after the first two adsorptions has been observed in every case where charcoal was used as adsorbent, and is quite definitely not due to experimental error. We have as yet no explanation of this curious effect.

The charcoal method, therefore, enables us to concentrate the whey preparation a further five times. The filtrate after four charcoal adsorptions is a clear, colourless solution of very high activity; and by half-saturation of this with ammonium sulphate, filtering off the precipitate, and drying *in vacuo*, we obtained the whole of the enzyme in the form of a preparation which was, as we expected, five times as active as the whey preparation, or 2500 times as active as milk.

(*b*) *Kaolin.* It was found that kaolin could also be used for the further purification of the whey preparation. In order to ascertain the best conditions for its use the factors influencing the adsorption of the enzyme by kaolin were studied.

The following experiments show how the adsorption depends on the p_H of the solution. 1 g. of a moderately active whey preparation was dissolved in 100 cc. of distilled water. The reduction time, under the conditions given above in the description of the charcoal experiment, was 2′ 40″. 10 cc. of this solution were measured into each of a series of test-tubes, and 1 cc. of acetic acid or sodium hydroxide of the strength given in Table II was added. The p_H of each solution was then determined colorimetrically, using a few drops

only. To each test-tube 0·5 g. of kaolin was added, the mixture was shaken thoroughly for 1 minute, and then filtered through a small Whatman filter-paper by suction. The enzyme and protein contents of each clear filtrate were determined in the manner previously described. The results are given in Table II.

Table II. *Adsorption of xanthine oxidase by kaolin at different p_H's.*

Enzyme solution	Acetic acid or NaOH	p_H	Reduction time	Enzyme activity	Relative protein content
10 cc.	1 cc. *N*/10 acid	3·4	29′	3·4	43·6
,,	,, *N*/20 ,,	4·2	43′	2·3	43·2
,,	,, *N*/40 ,,	4·7	66′	1·5	40·8
,,	,, *N*/80 ,,	5·0	91′	1·1	44·8
,,	,, water	5·6	59′	1·7	45·8
,,	,, *N*/100 NaOH	6·8	23′	4·3	56·0
,,	,, *N*/40 ,,	7·6	5′	20·0	62·0
,,	,, *N*/20 ,,	8·6	4′	25·0	63·5

Enzyme activity of original solution 37·5.

In slightly acid solution the xanthine oxidase is almost completely adsorbed by the kaolin, while nearly half the protein is left in solution. The most favourable p_H for the adsorption of the enzyme is 5·0. In slightly alkaline solution, on the other hand, only a small proportion of the enzyme is adsorbed. This marked change in the degree of adsorption with p_H in the case of the enzyme is in sharp contrast with the behaviour of the protein, as the adsorption of the latter is only slightly affected by the change in p_H. This effect is of interest as suggesting that the xanthine oxidase may contain a dissociable group which is responsible for the change.

Having determined the optimum p_H for adsorption we have next to find the minimum amount of adsorbent to effect complete adsorption. It was found that treatment of 50 cc. of a solution of the whey preparation (containing 1 g. of the latter, having an enzyme activity of 100, and adjusted to p_H 5·0) with 3 g. of kaolin removed about 85 % of the enzyme, and treatment of the resulting filtrate with a further 2 g. of kaolin brought the amount adsorbed up to 97 %. It was therefore concluded from this experiment that 5 g. of kaolin is sufficient for 50 cc. of a 2 % solution.

The data presented in Table II suggested that the adsorbed enzyme might be liberated from the kaolin by extraction at an alkaline p_H. This actually proved to be the case, and it was found that extraction with 1 % sodium carbonate solution gave the best results. Unfortunately, however, it was not found possible to extract more than 50 to 60 % of the enzyme adsorbed. Table III gives the results of extracting the 5 g. of kaolin obtained in the experiment described in the previous paragraph.

Table III. *The liberation of the adsorbed enzyme by* 1 % Na_2CO_3.

No. of extraction	Na_2CO_3 used cc.	Reduction time	Enzyme activity	Enzyme recovered %
1	20	0′ 51″	118·0	47·0
2	10	3′ 5″	32·5	6·5
3	10	10′ 0″	10·0	2·0

Although the kaolin treatment thus resulted in a loss of about 45 % of the enzyme the fall in the protein content was found to be considerably greater in proportion, having been reduced to less than one-eighth of its original value by this treatment; and on making a solid preparation in the usual way by half-saturation of the filtrate with ammonium sulphate it was found that it had an activity of between four and five times that of the whey preparation[1].

We may say, then, that the charcoal method gives a means of purifying the whey preparation without loss of enzyme, but can only be used for the removal of 80 % of the protein. On the other hand, a single kaolin adsorption and extraction is sufficient to give this degree of purification, and a repeated application of this treatment would undoubtedly give preparations of a very much higher activity, but unfortunately the 50 % loss of enzyme at each stage renders the method unsatisfactory.

The most active preparations we have obtained have been made by a combination of both methods. The following is a brief account of the procedure.

5 g. of the whey preparation were dissolved in 250 cc. of distilled water. This solution was treated four times in succession with 5 g. of charcoal in the manner described above. The filtrate thus obtained was slightly alkaline (p_H 7·8), and was therefore adjusted to p_H 5·0 by adding dilute acetic acid. To this solution were added 5 g. of kaolin, and after thorough mixing it was filtered by suction. This treatment with kaolin was repeated once more. The 10 g. of kaolin were transferred to a mortar and triturated with 100 cc. of 1 % Na_2CO_3 solution. On filtering, a clear solution was obtained. To this an equal volume of saturated ammonium sulphate solution was added, and after standing for 30 minutes the whole solution was centrifuged. The supernatant fluid was poured off and the precipitate collected on a hard filter-paper. (A hard paper should be used, as the material at this stage is very sticky.) After being squeezed well, it was dried in the vacuum desiccator over sulphuric acid. The yield was about 0·23 g.

In the process of preparation it is important to avoid prolonged contact with air as far as possible, as the purified enzyme is very easily destroyed in the moist state by oxygen. The dry preparation is perfectly stable, however, and can be kept in the desiccator for long periods.

The final preparation is very readily soluble in water, producing a perfectly clear solution. The activity is from 8 to 10 times that of the whey preparation, or about 4000 times that of milk. 1 mg. of the preparation gave a reduction time of 1′ 30″ at 13°, corresponding to 4 cc. of the fresh milk.

[1] Since the work described in this paper was completed a paper by Sbarsky and Michlin [1926] has appeared describing the further purification of their buttermilk preparation by a method essentially similar to that given here. The method consists in the digestion of their solid preparation for several days with dilute acid, the adsorption of the enzyme from the acid digest by kaolin, extraction of the enzyme from the kaolin by very dilute ammonia, and finally evaporation to dryness *in vacuo*. The activity of the final preparation was 2000 times that of milk. The figures show that here also there was a loss of about 45 % of the enzyme.

We are much indebted to Sir F. G. Hopkins for his advice and encouragement.

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CXLI. A RAPID COLORIMETRIC METHOD OF ESTIMATING PENTOSES.

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The basis of the estimations of pentoses now in use is the quantitative conversion of the pentose into furfural by boiling with dilute hydrochloric acid. The furfural so formed, being volatile in steam, passes over into the distillate (together with water and HCl), and is then estimated by one of several methods: Plimmer [1920]; Ling and Nanji [1921]; Youngburg and Pucher [1924]. While the method herein described also depends upon the estimation of furfural, it differs from the others in certain respects which make it very rapid and easy to execute.

Youngburg and Pucher [1924] have described a colorimetric method of estimating the furfural in the distillate which depends upon the well-known colour given by furfural in the presence of aniline acetate. Benzidine has now been found to give a much more intense colour with furfural than aniline, and (in my opinion) a colour much more easy to match against a standard. The colour given by benzidine is a deep crimson violet. It is, however, completely destroyed by water. This difficulty can easily be overcome by shaking the "unknown" furfural solution with benzene, in which furfural is much more soluble than in water, and, after partition has taken place, using the benzene portion for the furfural estimation. The "standard" solution of furfural in water must also be shaken with benzene under identical conditions. The colours are best matched when the "unknown" and "standard" are within 25 % of each other, but a rough idea of the strength of the "unknown" can be obtained over a much wider range. The reason for this is that the reagent (described below) is itself a light yellow and this makes the weaker solution appear more red and less violet than the stronger, because in the former one is looking through a deeper layer of the yellow reagent. This micro-method of estimating furfural is obviously capable of general application.

By taking advantage of the fact that furfural is freely soluble in benzene a very simple, easy method of estimating the pentoses has been devised (it has, however, some well-defined limitations). This involves no distillation, and requires only test-tubes and a water-bath in the way of apparatus.

Method.

The "standard" is made up as follows: 3 cc. 0·1 % arabinose, 3 cc. H_2O and 3 cc. conc. HCl are placed in a test-tube. 1 cc., 2 cc. or 3 cc. etc. of the "unknown" solution is placed in another test-tube and the volume made up to 6 cc. with water; 3 cc. of conc. HCl are then added. The tubes are placed in a water-bath at 100° for 2 hours. Each test-tube is closed by a cork through which passes a long piece of glass tubing which acts as a condenser. After 2 hours the tubes are removed from the bath and cooled, any drop in the glass tube being blown out. 4 cc. of benzene are added to each tube which is then shaken vigorously for 3 minutes. The test-tubes are then allowed to stand 30 minutes. There may be some black material in the layer of benzene above the "unknown," but this can be dispersed by spinning the tube between the hands when the black material sinks or sticks to the side. 2 cc. of the benzene layer are then withdrawn with a pipette and placed in a dry test-tube; it is essential that none of the aqueous layer be withdrawn with the benzene. 4 cc. of benzidine reagent are then added to each of the test-tubes. The colour takes some 15 minutes to develop, and the tubes may be matched any time between 30 minutes and 2 hours after adding the reagent. It is best to have each tube in duplicate. The duplicates may conveniently be mixed just before matching in the colorimeter. The number of estimations which can be carried out simultaneously is only limited by the space available for test-tubes in the water-bath.

This method is only capable of application (*a*) when there is no colouring matter extracted by the benzene from the "unknown," or at any rate not enough to interfere with the colorimetric estimation of the furfural; (*b*) when the pentose to be estimated is the same as the "standard," because all the pentose is not quantitatively converted to furfural by this method, and as different pentoses (and glycuronic acid) may be converted to furfural at different rates, equilibrium may not have been reached in all cases; (*c*) when the "unknown" does not contain enough proteins or buffering substances to decrease appreciably the concentration of HCl. This must be a matter for experiment in each case.

The benzidine reagent is made up as follows: 0·5 g. of benzidine is dissolved in 100 cc. of equal parts of absolute alcohol and glacial acetic acid. The solution is then filtered. It keeps for some time but it is best to make it up fresh once a week.

Some experimental results on a urine to which known amounts of arabinose had been added may now be given. The sample of urine used contained by this method 0·02 % of "pentose." Actually this substance was probably a mixture of pentoses and glycuronates, and to estimate its absolute amount one would have to distil the urine with HCl and estimate the furfural in the distillate. Solutions of arabinose in urine of the following strengths were then made up: (*a*) 1 %; (*b*) 0·4 %; (*c*) 0·1 %; (*d*) 0·05 %. 0·3 cc. of (*a*), 0·8 cc. of (*b*),

3·0 cc. of (*c*), 6·0 cc. of (*d*) were taken for each estimation, the "standard" being 3 cc. of 0·1 % arabinose, 3 cc. H_2O and 3 cc. conc. HCl. With the "standard" set at 20 the colorimeter readings were (*a*) 19·1; (*b*) 17·3; (*c*) 16; (*d*) 14·9, making the strength of arabinose by estimation:

(*a*) 1·05 − 0·02 = 1·03 %.
(*b*) 0·43 − 0·02 = 0·41 %.
(*c*) 0·125 − 0·02 = 0·105 %.
(*d*) 0·067 − 0·02 = 0·047 %.

SUMMARY.

A rapid and simple method of estimating pentoses is described involving no distillation and depending on a colour reaction between furfural and benzidine.

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CXLII. SOME OBSERVATIONS ON THE OXIDATION OF PHENOLS BY TISSUES AND ON THE SIGNIFICANCE OF SURFACES FOR BIOLOGICAL OXIDATIONS.

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(*Received July 30th, 1926.*)

Phenols, which are so important in the oxidations of plants, would be also of great importance for oxidation in animals, if, as Szent-Györgyi [1924; 1925, 1, 2] asserts, it could be proved that they increase lactic acid oxidation in mammalian muscle; for the conditions of lactic acid oxidation in mammalian muscle are still undefined, though of great significance for the physiology of muscle. Szent-Györgyi's conclusion that a co-enzyme is concerned in its oxidation and his suggestion that this co-agent may be a phenol are of much interest. The intention of the work to be described was to repeat the experiments of the above author in the hope of linking them with other work which is in progress in this laboratory. I must state in advance, however, that to judge from my own experiments as described in this paper it is not possible to increase the oxidation of lactic acid either by ice-water extract of muscle (which according to Szent-Györgyi contains the co-enzyme), or by *p*-phenylenediamine. Szent-Györgyi [1925, 1, 2] ground the muscle he employed with glass-powder and the powder was contained in the systems of which the oxygen uptake was determined. It is clear, however, that the introduction of a foreign surface of the kind may appreciably alter conditions in a system. The following experiments were therefore carried out:

(i) the effect of boiled ice-water extract on minced muscle;

(ii) the influence of some phenols on the oxygen uptake of muscle and of reduced glutathione;

(iii) the influence of various surfaces on the oxidation of phenols;

(iv) the oxidation of phenols in "mixed systems" such as Szent-Györgyi's.

I.

The muscle, pig's diaphragm, was treated carefully in accordance with Szent-Györgyi's method [1925, 1, 2]. The muscle, freed from sinew and fat, was minced with a chopper on a block, then washed in cool distilled water twice, from 15 to 20 minutes each time, filtered through linen and pressed between filter-papers and finally pounded in a mortar with glass-powder which had been previously washed ten times in distilled water. The oxygen

uptake of this muscle, so treated, was measured in Barcroft's differential manometer at 37°–38°, and the decoloration of methylene blue was observed anaerobically in Thunberg's tubes.

The ice-water extract was made in the following way: 10 g. minced muscle were mixed with 10 cc. water at 1°–3°, the whole put in ice for 1–2 hours and frequently stirred. It was then filtered through linen, squeezed and the coloured solution was boiled, filtered again through paper and made up to four times the amount of the original extract with a phosphate buffer solution of p_H 7·4.

Working with this glass-powder-muscle was not satisfactory, because, as Szent-Györgyi found, the results were not consistent (Table I); but working

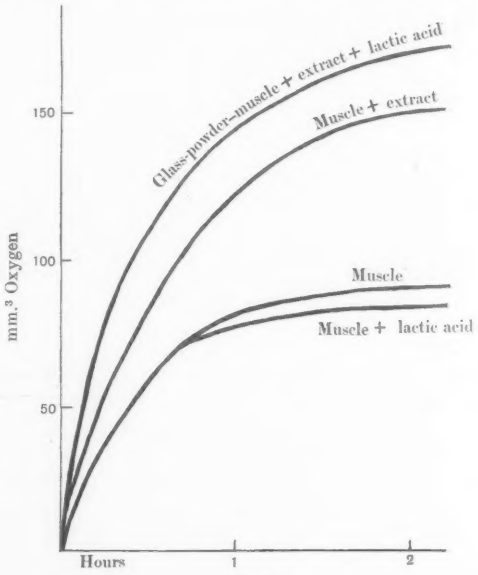

Fig. 1.

with the muscle without glass-powder the agreement was quite constant (cf. Table II). From Table II we see firstly that muscle washed as above always takes up some oxygen and in fairly constant amount, but only in phosphate solution, never in 0·9 % NaCl solution (unpublished experiments); secondly, that the muscle prepared in this way oxidises lactic acid constantly, though in small amounts which increase with the lactic acid concentration.

Table I shows that the oxygen uptake of the system muscle + glass-powder + lactic acid was indeed occasionally materially increased by the addition of the boiled water-extract; but we noticed that sometimes the oxygen uptake of the glass-powder-muscle was decreased by the addition of lactic acid (cf. Fig. 1).

Table I.

$37°$; $p_H = 7·4$.

Material	Duration of experiments $2\frac{1}{4}$ hrs.	$4\frac{3}{4}$ hrs.	$5\frac{1}{4}$ hrs.
	Oxygen uptake mm.³		
1 g. glass-powder-muscle	90	16	162
1 „ „ „ +2·5 cc. boiled water-extract	144	113	218
1 „ „ „ +0·3 cc. 0·25 *M* lactic acid	84	94	116
1 „ „ „ +2·5 cc. boiled water-extract +0·3 cc. 0·25 *M* lactic acid	176	208	315

Table II.

$38°$; $p_H = 7·4$.

Material	Duration of the experiments in hours: $3\frac{1}{2}$	$5\frac{1}{4}$	4	4	4	4	4	4	4	3	4	4	4	4
	Oxygen uptake mm.³													
0·7 g. washed muscle	40	50	40	35	35	58	35	51	48	31	61	52	60	60
0·7 „ „ +2·5 cc. extract	154	264*	164	160	266*	408**	134	—	—	—	—	—	—	—
0·7 „ „ +0·3 cc. 0·25 *M* lactic acid [0·025 *M*]	64	—	70	64	—	62	74	—	—	—	—	—	—	—
0·7 „ „ +2·5 cc. extr. +0·3 cc. 0·25 *M* lactic acid	196	—	211	152	—	462	136	—	—	—	—	—	—	—
0·7 „ „ +0·6 cc. 0·25 *M* lactic acid [0·05 *M*]	—	104	—	—	111	—	—	—	—	95	100	100	110	—
0·7 „ „ +2·5 cc. extr. +0·6 cc. 0·25 *M* lactic acid	—	268	—	—	347	—	—	—	—	—	—	—	—	—
0·5 „ „ +0·9 cc. 0·25 *M* lactic acid [0·75 *M*]	—	—	—	—	—	—	—	138	133	—	152	—	—	—
0·5 „ „ +0·6 cc. 0·5 *M* lactic acid [0·1 *M*]	—	—	—	—	—	—	—	—	—	—	—	—	195	184
0·5 „ „ +0·6 cc. 1·0 *M* lactic acid [0·2 *M*]	—	—	—	—	—	—	—	—	—	—	—	239	—	—

The values in brackets denote final concentrations of lactic acid; the oxygen uptake was complete; the extract was diluted 1 in 4; extr.* 1 in 3; extr.** 1 in 2.

In anaerobic experiments it was observed that the capacity for methylene blue reduction was greatly increased by adding lactic acid to the mixture of muscle and glass-powder, but the increase was very small when lactic acid was added to muscle without glass-powder (cf. Table III). This result will be discussed later.

Table III. *Anaerobic experiments.* p_H 7·4; 37°.

Meth. blue 1 : 2000	Muscle (washed)	Lactic acid 0·3 *M*	Ice-water extract boiled	Phosphate buffer	Decoloration time in minutes
cc.	g.	cc.	cc.	cc.	
0·3	1 with glass-powder	0·25	—	1·00	6
0·3	1 with glass-powder	—	—	1·25	64
0·3	1 with glass-powder	—	1	0·25	3
0·3	1 with glass-powder	0·25	1	—	4
0·3	0·7 without glass-powder	0·25	—	1·0	14
0·3	0·7 without glass-powder	—	—	1·25	14
0·3	0·7 without glass-powder	—	1	0·25	4·5
0·3	0·7 without glass-powder	0·25	1	—	4·5
0·5	1 without glass-powder	—	—	4·5	25
0·5	1 without glass-powder	1·0	—	3·5	22

Meanwhile, it is well to point out that the aqueous extract of muscle is very rich in H-donators; it gives, of course, a strongly positive nitroprusside reaction. The extract itself is not autoxidisable under the conditions which obtain in the Barcroft manometer, but takes up oxygen under the catalytic influence of haemoglobin; *e.g.* 2 cc. extract + 0·5 cc. haemoglobin solution (0·5 %) + 2·5 cc. buffer solution take up 94 mm.^{3} O_2 in 5 hours, but 0·5 cc. haemoglobin solution (0·5 %) + 2·5 cc. buffer take up under the same circumstances 30 mm.^{3}; the extract alone is not autoxidisable.

As it was not possible to obtain consistent results with the glass-powder-muscle, I made the following experiments on muscle, prepared in the way stated above, but without glass-powder.

Naturally it seemed possible that the haemoglobin present in the aqueous solution might play a part in the effects of the extract on muscle; I therefore made some experiments on the influence of haemoglobin on the oxygen uptake by muscle (cf. Table IV). The haemoglobin preparation I owe to the kindness of Mr G. S. Adair; from this haemoglobin I made methaemoglobin by adding a trace of potassium ferricyanide and removing the latter by dialysis.

Table IV. 37°; $p_H = 7{\cdot}4$.

0·5 g. washed muscle	Haemoglobin	Methaemoglobin	0·5 g. muscle + haemoglobin	0·5 g. muscle + methaemoglobin	Duration of the experiments
Oxygen uptake mm.^{3}					
60	12*	20	195*	199	5 hours (complete)
54	15**	18	130**	118	4 „ (nearly complete)
48	17***	20	215***	222	5 „ (complete)

Concentration of methaemoglobin 2 %; of haemoglobin* 0·1 %; of haemoglobin** 0·5 %; of haemoglobin*** 2 %.

It will be seen that haemoglobin and methaemoglobin both catalyse the oxygen uptake of washed muscle to the same extent, as Robinson [1924] also found when working on unsaturated fatty acids. That this effect of methaemo-

globin is purely catalytic follows from the fact that the haemoglobin concentration is of no influence in this process. But in four experiments I found no increase of lactic acid oxidation on the addition of haemoglobin or methaemoglobin. I may further point out that some preliminary experiments showed that methaemoglobin reacts with glutathione. That methaemoglobin may be reduced by GSH[1] has been incidentally pointed out by Holden [1924]; I have confirmed his observation as follows: 1 cc. 8 % methaemoglobin solution + 50 mg. GSH ceased to show the characteristic absorption bands within 2–2½ hours at 37°. This reduction is also brought about by washed muscle added to the methaemoglobin solution.

II.

I have made a number of experiments dealing with the influence of *p*-phenylenediamine on the oxygen uptake of washed muscle (studied first by Batelli and Stern [1912]), since Szent-Györgyi states that this substance increases the oxidation of lactic acid by muscle. I found in fifteen consistent experiments that this is not the case. Fig. 2 shows that the oxygen uptake

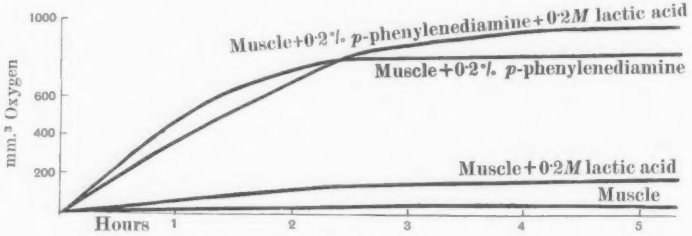

Fig. 2.

of the systems, muscle + *p*-phenylenediamine and muscle + *p*-phenylenediamine + lactic acid, is additive. In all the experiments the oxidation of muscle + *p*-phenylenediamine is at first diminished by addition of lactic acid and after 2–2½ hours increased. Both influences depend on the concentration of the lactic acid, and increase with increase of the latter. So there seems to be no probability that the oxidation of lactic acid by muscle is increased as the result of the presence of *p*-phenylenediamine.

It was further necessary to try to make clear the mechanism of the oxidation of *p*-phenylenediamine by muscle and to compare it with the oxidation of other aromatic substances, such as phenols. Table V shows that in the concentrations given the oxygen uptake of *p*-phenylenediamine in the presence of muscle is nearly proportional to its concentration. The diamine in the concentrations employed has itself only a small uptake (cf. Table VI). This uptake is not affected by *M*/500 KCN.

[1] GSH = reduced glutathione.

Table V. $p_H = 7{\cdot}4$; 38°.

Material	Oxygen uptake** mm.³
0·5 g. washed muscle	61
0·5 „ „ in 0·001 % *p*-phenylenediamine*	102
0·5 „ „ in 0·0035 „ „ „	218
0·5 „ „ in 0·05 „ „ „	300
0·5 „ „ in 0·1 „ „ „	573, 573, 552, 550, 550
0·5 „ „ in 0·15 „ „ „	800
0·5 „ „ in 0·2 „ „ „	1010, 1020
0·5 „ „ in 0·33 „ „ „	1510

* Final concentration; ** oxygen uptake in all experiments complete.

Table VI. $p_H = 7{\cdot}4$; 38°.

The concentrations given denote *final* concentrations.

Material	Oxygen uptake mm.³	Duration of the experiments
0·5 g. washed muscle	61	
0·5 „ „ +0·1 % *p*-phenylenediamine	573	5 hours
0·5 „ „ +0·33 % GSH*	182	(nearly complete)
0·5 „ „ +0·1 % *p*-phenylenediamine + 0·33 % GSH	773	
0·1 % *p*-phenylenediamine	39	
0·1 % *p*-phenylenediamine	29, 35	2 hours
0·1 % „ „ +0·33 % GSH	208, 196	(complete)
0·33 % GSH	150, 150	
0·2 g. muscle-powder	24	
0·2 „ „ +0·1 % *p*-phenylenediamine	69	4 hours
0·2 „ „ +0·33 % GSH	226	(complete)
0·2 „ „ +0·1 % *p*-phenylenediamine + 0·33 % GSH	210	

* The GSH preparation was in fact only 40 % reduced.

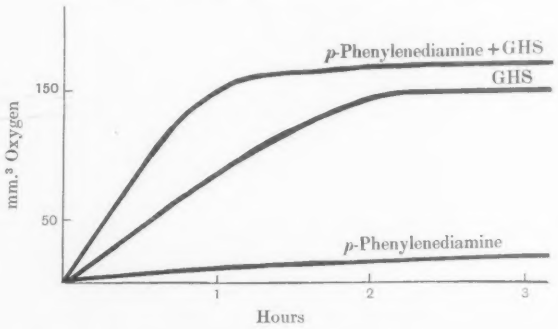

Fig. 3.

Very peculiar are the relations of *p*-phenylenediamine to GSH. Autoxidation of GSH is considerably accelerated (Fig. 3) by 0·1 % *p*-phenylenediamine in the absence of muscle and perhaps also increased in amount; but the oxygen uptake of the system muscle + GSH is perceptibly augmented by *p*-phenylenediamine (cf. Table VI). As follows from some observations carried out conjointly with Dr M. Dixon, however, it seems that a compound of these two substances is formed; because, since the anaerobic decoloration of methylene blue by GSH is likewise accelerated by *p*-phenylenediamine, it seems as though *p*-phenylenediamine activates the hydrogen of the GSH group. Methylene blue (0·5 cc.) + 0·1 cc. 1 % GSH + 4·8 cc. buffer solution ($p_H = 7{\cdot}5$) was

decolorised in 100 minutes; whilst 0·5 cc. methylene blue + 0·1 cc. GSH + 0·5 cc. 1 % *p*-phenylenediamine + 4·3 cc. buffer solution ($p_H = 7·5$) took 73 minutes to become decolorised. Both experiments were performed at 37°. No such effect was observed in aerobic and anaerobic experiments with catechol, quinol, *p*-cresol, or adrenaline.

In order to determine whether the effect of the *p*-phenylenediamine was due to traces of iron contained in it accelerating the reaction [see Toda, 1926], we investigated the effect of cyanide as follows:

Contents of tubes	Reduction time min.
0·5 cc. meth. blue + 0·2 cc. GSH (1 %) + 1·3 cc. buffer ($p_H = 7·4$–$7·6$)	68
0·5 „ „ + 0·2 „ „ + 0·9 „ + 0·4 cc. *M*/100 KCN	300
0·5 „ „ + 0·2 „ „ + 0·4 „ + 0·5 cc. *p*-phenylenediamine (1 %) ...	28
0·5 „ „ + 0·2 „ „ + 0·4 cc. *M*/100 KCN + 0·5 cc. *p*-phenylenediam. (1 %)	58

GSH = glutathione 40 % reduced. All solutions were carefully neutralised before use.

It will be seen that the cyanide has decreased the velocity of reduction by the GSH to less than one quarter of the normal, in agreement with the results of Toda. On the addition of *p*-phenylenediamine the reducing power is again restored, and there is even a slight acceleration in spite of the presence of cyanide. It is quite clear, therefore, that *p*-phenylenediamine is able to activate the SH group quite apart from any traces of iron which it may contain. *p*-Phenylenediamine even acts like iron in this case.

We further observed that the reduction potential (measured with a gold electrode) of mixtures of GSH and *p*-phenylenediamine was much higher than that of either substance alone; *e.g.* reduction potential against saturated calomel electrode (20°) of ·00003 *M* of our preparation of GSH (= ·0000075 *M* GSH):—55 millivolts; 0·16 % *p*-phenylenediamine:—80 millivolts; mixture of both:—121 millivolts; all substances were dissolved in phosphate buffer of p_H 7·4.

In connection with these observations it is of interest to note that McCance [1925] found that catechol, which is unable to reduce methylene blue, forms compounds with certain amines and these compounds rapidly reduce methylene blue.

III.

For the promotion of chemical reactions by living tissues two factors must be taken into consideration: (1) chemical factors involving the influence of certain substances or groups; (2) physical factors, *i.e.* possibilities of orientation of these molecules: but how such an orientation of molecules, *e.g.*, on surfaces, may modify the possibilities of chemical reaction ("Aktivierung") is not yet well understood.

Table VII and Figs. 4 and 5 show the effect of different surfaces on the oxidation of phenols, etc. The oxidation of *p*-phenylenediamine, which is slightly autoxidisable in the concentrations used by us, is catalysed by washed muscle and charcoal considerably, and by the first not very much more than by the second. It is not affected by coagulated muscle-powder, which it also

fails to colour. *p*-Phenylenediamine, moreover, has no influence on the muscle-powder + glutathione system (Table VI). The oxidation of 0·1 % quinol under the same circumstances is hardly catalysed by charcoal, but by washed muscle it is catalysed two and a half times as much as is *p*-phenylenediamine, whilst 0·1 % catechol is catalysed by both charcoal and muscle to the same extent, namely, about half as much as *p*-phenylenediamine.

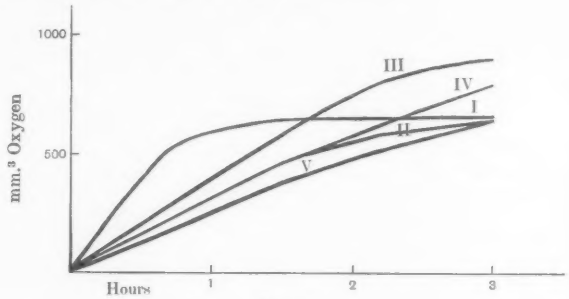

Fig. 4.

I. Washed muscle +1 % *p*-phenylenediamine
II. „ „ (60°) +1 % „
III. „ „ +1 % quinol
IV. „ „ (46°) +1 % „
V. „ „ (60°) +1 % „

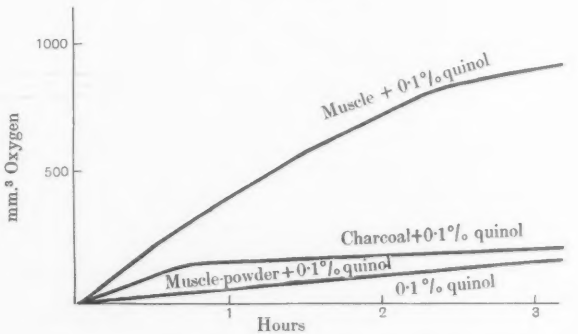

Fig. 5.

Table VII. 38°, $p_H = 7·4$; 5 hours.

	Without addition	+Muscle-powder	+Charcoal	+Washed muscle
	Oxygen uptake mm.³			
0·1 % *p*-phenylenediamine	38	69	398	550
0·1 % quinol	192	200	235	1056
0·1 % catechol	100	182	284	258

If the muscle is washed to excess (ten times), it has more effect on the oxidation of *p*-phenylenediamine; thus 0·5 g. twice washed muscle + 0·1 % *p*-phenylenediamine takes up 555 mm.³ (cf. Table V), but 0·5 g. ten times

washed muscle + 0·1 % *p*-phenylenediamine took up in one experiment 727 mm.³, in another 850 mm.³; washing therefore seems to increase this catalytic activity of muscle. If this muscle be warmed to 60° for 1 hour, the catalytic effect is diminished without lessening the final uptake; the delay of the uptake is still greater in the case of quinol. This can perhaps be explained by the alteration of the tissue by loss of water (cf. Table VIII). Heating to 80°, on the other hand, nearly completely destroys the activity.

Table VIII. 5 hours, 38°.

Material	Oxygen uptake mm.³	Remarks
0·1 % *p*-phenylenediamine	39	
+1 g. muscle washed 10 times	727*	
+0·75 g. „ „ warmed to 46° for 1 hour	780*	Loss of weight 28 %; slow oxidation
+0·5 „ „ warmed to 60° for 1 hour	730*	Loss of weight 48 %; slow oxidation (cf. Fig. 4)
+0·4 „ „ coagulated at 80°	100*	Loss of weight 58 %
+0·2 „ „ „ „ „ and dried by alcohol	68*	„ „ 80 %
0·1 % quinol	192	
+1 g. muscle washed 10 times	1056*	
+0·75 g. „ „ warmed to 46° for 1 hour	979	Slow oxidation
+0·5 „ „ „ to 60° for 1 hour	857	„ „
+0·2 „ „ coagulated at 80°, dried by alcohol ...	216*	

In the experiments * the oxygen uptake was complete.

From these experiments the following conclusions concerning the biological oxidation of these phenols seem justified. The oxidation of *p*-phenylenediamine is accelerated on surfaces; washed muscle and charcoal show little difference in this respect. Consequently, for this catalysis only an adsorbing surface is necessary, it is therefore for the most part physical. For oxidation of quinol a chemical activation given by the non-coagulated muscle is the essential process, and charcoal and muscle-powder have no effect; also for oxidation of catechol there seems to be necessary such a chemical activation, which however does not occur in muscle.

Table IX. $p_H = 7{\cdot}4$; 38°.

Washed muscle g.	Final concentration of lactic acid	Final concentration of *p*-phenylenediamine %	Charcoal g.	Oxygen uptake (mm.³) 5 hrs.	4 hrs.	4 hrs.	4 hrs.
1	—	—	—	50	50	50	—
1	0·2 *M*	—	—	230	—	—	—
1	0·175 *M*	—	—	—	158	—	—
1	0·2 *M*	—	0·2	30	—	—	—
1	—	0·1	—	520	549	—	—
1	0·2 *M*	0·1	—	720	—	—	—
1	—	0·1	0·2	440	—	416	—
1	0·2 *M*	0·1	0·2	554	—	—	—
1	0·175 *M*	—	0·2	—	65	—	30
1	0·175 *M*	0·1	—	—	—	—	684
1	0·175 *M*	0·1	0·2		496	510	484

IV.

In order to make my results more comparable with those of Szent-Györgyi I made certain further investigations, in which muscle and an adsorbing surface were rivals for the phenol. In these experiments I put in one vessel of the manometer 1 g. washed muscle + 0·2 g. charcoal + the substances to be tested, and in the other the same amount of charcoal. Table IX gives the details of some results on these lines which showed a fairly good agreement.

The oxygen uptake of the system muscle + charcoal + *p*-phenylenediamine appears to be increased by adding lactic acid to a greater extent than additive effects would account for, but lactic acid oxidation by muscle is greatly hindered by charcoal, just as we have already seen it to be hindered by glass-powder. Therefore, the most probable explanation of Szent-Györgyi's inconstant results, showing that the oxidation of lactic acid by muscle is increased by *p*-phenylenediamine, seems to be that added lactic acid, otherwise adsorbed by the glass-powder, is displaced from the glass-powder surface by *p*-phenylenediamine and so may be oxidised by the muscle. Thus, in his experiments there was a rearrangement of the surfaces involved in his oxidising systems which disturbed the true physiological relations and obscured the real factors involved in the oxidation of lactic acid. That no more lactic acid is oxidised by muscle as the result of the presence of Szent-Györgyi's ice-water extract has been shown above. That the presence or absence of *p*-phenylenediamine is immaterial is evident from Table X. In two manometers hanging side by side we investigated two identical mixtures; in one manometer was added, as usual, 20 % KOH to absorb CO_2, but none in the other; in this manner we could measure roughly the CO_2 formed by the oxidation; as by the oxidation of *p*-phenylenediamine no CO_2 is formed, the whole measured amount of CO_2 (apart from that produced by the muscle alone, which will be the same in all experiments) is produced by the oxidation of lactic acid.

Table X. 38°; $p_H = 7{\cdot}4$–$7{\cdot}6$.

	Washed muscle + lactic acid			Washed muscle + lactic acid + *p*-phenylenediamine			Washed muscle + lactic acid + *p*-phenylenediamine + charcoal		
min.	mm.³ O_2	O_2–CO_2	CO_2	mm.³ O_2	O_2–CO_2	CO_2	mm.³ O_2	O_2–CO_2	CO_2
17	31	8·5	22·5	188	129	59	166	104	62
28	53	20	33	296	222	74	232	154	78
55	103	48	55	498	406	92	332	208	124
70	—	—	—	572	474	98	368	236	132
131	185	85	100	674	549	125	448	304	144
255	240	114	126	710	591	119	526	366	160

In these experiments were used washed muscle, 1 g.; charcoal, 0·2 g. The final concentration of lactic acid was 0·2 *M*, of *p*-phenylenediamine, 0·1 %.

Consequently we see that the oxidation of lactic acid is not altered by adding *p*-phenylenediamine, but there is an increase if charcoal is added.

SUMMARY.

1. Szent-Györgyi's results on the activation of the oxidation of lactic acid by muscle by addition of ice-water extract of the muscle and of *p*-phenylenediamine could not be confirmed.

2. The effects observed by Szent-Györgyi are shown to be due to a displacement of lactic acid from the surface of the glass-powder present in his muscle preparations by *p*-phenylenediamine, thus enabling it to be oxidised by the tissue.

3. Haemoglobin and methaemoglobin catalyse the oxygen uptake of washed muscle but do not affect lactic acid oxidation.

4. *p*-Phenylenediamine is oxidised by washed muscle and by charcoal, but not by coagulated muscle. Heating washed muscle to 60° for an hour diminishes the activity, heating to 80° entirely destroys it. *p*-Phenylenediamine accelerates aerobically and anaerobically the oxidation of reduced glutathione, probably by forming a compound with it. The oxidation of *p*-phenylenediamine is essentially a surface catalysis; the oxidation of quinol and catechol, on the other hand, is not accelerated by surfaces such as charcoal.

I wish to express my best thanks to Sir F. G. Hopkins for receiving me so generously in his laboratory, and to him and his co-workers for their kind help; I have also to thank the Universitätsbund Göttingen and the Kuratorium der Universität Göttingen for travelling grants.

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CXLIII. EXPERIMENTS ON "STRICT" ANAEROBES.

1. THE RELATIONSHIP OF *B. SPOROGENES* TO OXYGEN.

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In the course of an investigation into the nutritional requirements governing the growth of the "strict" anaerobes, as typified by *B. sporogenes*, two factors have come to light. The first of these conditions the relation of the cell to oxygen; this alone will be dealt with in the following communication. The second is concerned with nutrition and continued reproduction and will be dealt with in a later communication.

Since the discovery by Pasteur [1861] of the existence of organisms "vivant sans gas oxygène libre" the relationship of the true anaerobes to oxygen has been obscure. Pasteur himself considered that air killed them.

The toxic effect of oxygen has been generally accepted since Pasteur's day and numerous attempts have been made to explain this phenomenon. Recently McLeod and Gordon [1923] have observed that anaerobes contain little or no catalase, and they have attributed the toxic effect of oxygen to the production of hydrogen peroxide with which the cell, in the absence of catalase, has been unable to cope.

Callow [1923] has expressed the same conviction and supports it by showing that the anaerobes possess little or no catalase so that "in the absence of catalase the hydrogen peroxide would accumulate and kill the cell." She has stated, however [1924], that factors other than lack of catalase may play a part with the anaerobes. Sherman [1926] has noted that certain obligate anaerobes possess catalase.

Avery and Morgan [1924] find that the presence of sterile unheated plant tissues in a plain broth induces the aerobic growth of certain anaerobes and attribute the effect to decomposition of peroxide by the catalase and peroxidases of the plant. They consider that the failure of Callow and of McLeod and Gordon to obtain aerobic growth of anaerobes in media containing catalase is due to the possibility that "the complete oxidation-reduction system in the fresh plant tissue is able to decompose toxic peroxides of an order unaffected by catalase alone." McLeod and Gordon [1925, 2] assert that because bacteria possess reducing powers they are capable of giving rise to hydrogen peroxide and give indirect evidence [1925, 1] for the formation of this peroxide during the aerobic growth of some anaerobes.

This interpretation of the effect of oxygen is not accepted by Novy [1925] who has observed that the aerobic growth of anaerobes can be effected in an *h* tube by a distant culture of *B. subtilis* or by distant potato, both of which reduce the tension of oxygen by their aerobic respiration until an oxygen-free environment is established. The anaerobes then multiply. Thus, when oxygen is present in a medium inoculated with an anaerobe, proliferation does not occur; if oxygen be then removed mechanically, as by reduction of tension, multiplication of the organisms begins.

Hence the failure of anaerobes to grow aerobically cannot be due to a lethal action of oxygen or to the suicidal production of hydrogen peroxide; they appear rather to be in a state of suspended animation and apparently, from Novy's experiments, await only removal of the oxygen to multiply once more. The initial problem, therefore, of determining why oxygen exerts an inhibitory influence on the anaerobes still remains unsolved.

Novy suggests that the strict anaerobe depends for its proliferation on an enzyme—an anaerase—which can only work in the absence of oxygen. The suggestion is not very satisfactory for it postulates the existence of an enzyme which must be of such a nature that it becomes oxidised, or functionless, in the presence of oxygen and reduced, or capable of re-functioning, when the oxygen is removed—even when the process of removal is mechanical as by reduction of tension. We have no evidence for the existence of such an enzyme and later we will give results which make the suggestion untenable.

Preliminary observations.

As is well known, the growth of *B. sporogenes* on the usual nutrient media is accompanied by an offensive smell, partially, if not wholly, due to the formation of a number of sulphydryl compounds. We have performed numerous experiments in connection with the nutrition of the organism and we have found generally that the growth of the organism is accompanied by substances containing the —SH group[1], this either being liberated from, or present initially in, the medium.

Our attention was focussed in the first place on the significance of the —SH group in connection with the growth of *B. sporogenes*, by the following observation. A culture of the organism in tryptic broth had been allowed to stand exposed to air and room temperature for several days, after which time the culture had almost entirely lost its smell and ability to give a nitroprusside reaction. Inoculation of two drops of the culture into a freshly autoclaved tryptic broth medium, followed by incubation under strictly anaerobic conditions at 37°, failed to produce a growth within 20 hours, whilst the same inoculation into a freshly autoclaved tryptic broth containing 0·1 % neutralised cysteine hydrochloride resulted in a profuse growth of the organism. Further investigation showed that this property of inducing rapid growth of *B. sporogenes* was shared not only by cysteine but by thioglycollic acid and glutathione.

[1] As detected by the nitroprusside reaction.

Very small concentrations of these substances were equally effective and it was clear that the phenomenon was connected with the —SH group and not with any nutritional effect of the molecules as a whole. *B. sporogenes*, it may be added, fails to develop in a medium whose carbon or nitrogen is solely supplied by cysteine.

In our experimental work we have almost entirely confined ourselves to the use of cysteine to provide the —SH group. It is easily prepared in a state of purity and, in the form of its hydrochloride, is stable. It is easier to handle than thioglycollic acid, whilst the preparation of glutathione is too laborious and expensive to make this substance convenient for bacteriological purposes.

Media employed.

Tryptic broth was made according to Cole and Onslow's method [1916] and was used at a concentration containing 3 mg. N per cc. For work with *B. sporogenes* it was made up (with NaOH) to an initial p_H of 7·8.

The crude cysteine hydrochloride (prepared from hair) was recrystallised from alcohol and chloroform. It was added to tryptic broth so as to bring the concentration to 0·1 %, made up to p_H 7·8 with sodium hydroxide and immediately autoclaved. It will be convenient to refer to a tryptic broth medium as TB and to a tryptic broth medium containing 0·1 % cysteine hydrochloride as TB—CSH. The media were inoculated usually not longer than 3 hours after their preparation and sterilisation. Cysteine, it should be observed, oxidises fairly rapidly at p_H 7·8 and it is desirable to inoculate the media as quickly as possible after the preparation and sterilisation. Control experiments to exclude the possibility of contamination having occurred were always performed. 5 cc. of medium per tube were usually employed.

Exp. 1. Two or three drops of an 18-hour culture of *B. sporogenes* in tryptic broth were inoculated into TB and TB—CSH and incubated aerobically at 37°. After 18 hours there was no growth in TB but a heavy one in TB—CSH. If a large inoculation of a fresh culture of *B. sporogenes* be sown into TB, growth may occur aerobically, but this, we find, is generally associated with the fact that with a large inoculation a considerable quantity of —SH[1] is transferred along with the culture to the new medium. If little or no —SH is carried over, aerobic growth of *B. sporogenes* does not occur; good aerobic growth, on the other hand, takes place in TB—CSH. This phenomenon will be referred to in more detail later. Trenkmann has shown [1898] that the presence of alkali sulphide will induce aerobic growth of anaerobes.

Cysteine, thus, offers a contrast to catalase, which will not induce aerobic growth of the anaerobes. If the suggestion be right that the failure of catalase to induce aerobic growth is due to its inaccessibility to the peroxide formed within the cell, then it can be maintained that the success of cysteine is due either (1) to its preventing oxygen reaching the cell or (2) to its ability to reduce intracellular peroxide.

[1] It is very convenient to speak of —SH as though it were a separate entity.

Exp. 2. A culture of *B. sporogenes* in TB was exposed to pure oxygen for 6 hours at 37° and two drops were inoculated into TB and TB—CSH respectively. After 18 hours' incubation under strictly anaerobic conditions, the TB had failed to produce a growth whereas a prolific one was present in TB—CSH. These were then incubated anaerobically for a further 24 hours, at the end of which time a copious growth was present in TB.

On the peroxide theory it can be argued that exposure to oxygen has produced peroxide either in the organism or in the culture medium; hence no growth occurs in TB but a good one is present in TB—CSH where the cysteine removes the peroxide. But since growth occurs in TB—CSH it is clear that the effect of the oxygen (or peroxide produced by oxygen) is not lethal. Again, in TB alone growth developed after 42 hours' anaerobic incubation. This must mean on the peroxide theory that the peroxide produced by exposure to oxygen is simply removed by the broth itself, the effect of the presence of cysteine being to hasten this removal. But it is clear that the peroxide produced by exposure cannot have been fatal, otherwise no growth would have developed whether —SH were present or not and however long the incubation.

It may be urged that the effect of the oxygen has been to kill a very large proportion of the organisms, the long lag in growth in TB being due to the slow development of the survivors. This does not account for the fact, however, that the latent period almost entirely disappears in presence of CSH.

Exp. 3. 100 cc. of TB—CSH were placed in ten test-tubes, sown with a fresh culture of *B. sporogenes* and incubated aerobically for 24 hours. A copious growth in each tube resulted. The cultures were poured into sterile centrifuge tubes which were then covered with guttapercha caps, centrifuged and the crop of *B. sporogenes* washed once with sterile saline. It was then emulsified in 50 cc. saline. 20 cc. of this emulsion were placed in a small sterile wash-bottle and filtered air was bubbled through at room temperature for 4 hours. After this period two drops of the emulsion were inoculated into TB, TB—CSH and TB containing 1 % glucose (the glucose having been autoclaved separately). After 20 hours' anaerobic incubation, no growth was apparent in TB or TB-glucose but a profuse one in TB—CSH.

Exp. 4. An emulsion of *B. sporogenes* was made up as in the previous experiment, 10 cc. placed in a sterile wash-bottle and oxygen passed through the emulsion at room temperature for 6 hours. Three drops were inoculated into TB and TB—CSH. After 16 hours' anaerobic incubation there was little, if any, growth in TB but a prolific one in TB—CSH. Another 10 cc. of *B. sporogenes* were treated in a similar way with nitrogen and another with hydrogen. Both these emulsions after this treatment gave a good growth in TB, after 16 hours, as well as in TB—CSH. To another 10 cc. of the initial emulsion of *B. sporogenes*, neutral sterile cysteine hydrochloride was added to a concentration of 0·1 % and oxygen bubbled through for 6 hours. Anaerobic subculture showed, in 16 hours, a questionable growth in TB but a prolific one in TB—CSH.

Exp. 5. A culture of *B. sporogenes* in TB—CSH was bubbled with oxygen for 7 hours at room temperature. Subculture showed, after 18 hours' anaerobic incubation, a slight growth in TB but an excellent one in TB—CSH.

We may conclude quite definitely from the foregoing results that (1) oxygen is not fatal to *B. sporogenes*, (2) if oxygen gives rise to peroxide, this is only produced at a concentration small enough to affect proliferation but not to kill the organism. So far our results are in agreement with Novy's observations.

Exp. 6. 50 cc. of an emulsion of *B. sporogenes* was prepared as before and through this oxygen was passed for 24 hours at 37°. This emulsion was then divided into four parts, *A*, *B*, *C* and *D*, which were treated as follows:

(*A*) a rapid stream of hydrogen was run through to remove free oxygen;

(*B*) a stream of hydrogen sulphide was run through for 4 hours to remove oxygen and possibly peroxides. Hydrogen was finally run through to remove hydrogen sulphide;

(*C*) sterile neutral cysteine hydrochloride was added to a concentration of 0·1 % and the emulsion kept anaerobically at 37° for 4 hours. It was then centrifuged to remove —SH and the deposit of organism made up to the initial volume with sterile saline;

(*D*) the same procedure as in (*C*) was adopted but thioglycollic acid was substituted for cysteine.

The four emulsions were then inoculated into TB and TB—CSH and incubated anaerobically. The growths were examined after 18 hours and 66 hours. They are noted in Table I.

Table I.

Medium	TB	TB—CSH	TB	TB—CSH	TB	TB—CSH	TB	TB—CSH
Inoculant	*A*	*A*	*B*	*B*	*C*	*C*	*D*	*D*
Growth after 18 hours	0	+ + +	0	+ + +	0	+ + +	0	+
Growth after 66 hours	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +

These results show that the effect of prolonged exposure of *B. sporogenes* to oxygen is to cause a retardation of growth in TB (*i.e.* no growth in 18 hours but positive growth in 66 hours).

Removal of free oxygen from the organism mechanically as by means of hydrogen or chemically as by an —SH group does not immediately restore the cell to its initial condition. Oxygen must therefore affect the organisms in some way which is not *immediately* reversed by the removal of the oxygen. Novy's suggestion, which implies that the removal of free oxygen will result in immediate restoration of the anaerase and hence of the power of the organism to proliferate is, therefore, not supported.

The retardation of growth brought about by oxygen is not due to the presence of peroxides in the cell for the preliminary treatment of the oxygenated organism with —SH, which would have removed such substances, had no pronounced effect in restoring the immediate growth of the organism.

Now, since the lack of growth of the oxygenated organism in TB during the first 18 hours is not due to a lethal action of the oxygen, nor to the presence

of oxygen or peroxides in the inoculant, it follows, since good growth occurs in the same medium after 66 hours that, during the long latent period, either the inoculant or the medium has changed towards a condition favourable to anaerobic growth, and that the presence of —SH initially (as in TB—CSH) either greatly accelerates this change or, indeed, is itself responsible for the change. It is unlikely that tryptic broth alone will alter its composition during anaerobic incubation at 37°; the change, therefore, must be due to the inoculant. Since we know (1) that the presence of very little —SH will almost entirely remove the long latent period, and (2) that the growth of *B. sporogenes* on TB generates —SH compounds so that it is evident the organism possesses a proteolytic mechanism capable of producing —SH from TB, it appears reasonable to assume that the long latent period exhibited by the oxygenated organism is associated with the slow production of an —SH body, this being produced by the proteolytic activity of the inoculated cells and proliferation occurring immediately this —SH has reached a definite concentration. If this assumption is true we should be able to observe a powerful effect of —SH in a medium which initially contains no organic sulphur. For experiment we have used a digest of gelatin as one which initially is practically devoid of organic sulphur, and we have compared it with a digest of caseinogen (prepared similarly).

Exp. 7. Gelatin and caseinogen were completely hydrolysed with 20 % sulphuric acid for 30 hours, the sulphuric acid removed by baryta and the two digests neutralised and made up into stock solutions. They were used at concentrations containing 3 mg. N per cc. and at an initial p_H of 7·8. Phosphates and a little sulphate were added to them. Table II shows the effect of 0·1 % cysteine hydrochloride on the anaerobic growth of *B. sporogenes* in each medium. Two drops of a fresh culture of the organism were inoculated.

Table II.

Medium	Gelatin digest	Gelatin digest + CSH	Caseinogen digest	Caseinogen digest + CSH
Anaerobic growth after 16 hours	0	+ + +	+ + +	+ + +

Thus in a medium such as an acid digest of gelatin containing practically no organic sulphur, from which the cell can manufacture —SH, no anaerobic growth occurs unless —SH is artificially added, whereas in acid digest of caseinogen which—like tryptic broth—contains no initial —SH but a source of organic sulphur from which the organism can make it, good anaerobic growth occurs. An addition of CSH, however, supplies the necessary factor to the acid digest of gelatin.

Table III shows the effect of inoculating *B. tetanomorphus* into gelatin digest and tryptic broth in the presence and absence of either cysteine or of 1 % glucose. (The glucose was autoclaved separately.)

Table III.

Medium	Gelatin digest	Gelatin digest +CSH	Gelatin digest +glucose	TB	TB+CSH	TB +glucose
Anaerobic growth after 3 days ...	0	++	0	+++	+++	+++

The influence of —SH appears to be much greater than that of glucose. We have observed, however, that the addition of glucose to a gelatin digest at p_H 7·8 with subsequent autoclaving does produce a medium fairly favourable to the anaerobic growth of *B. sporogenes*. In performing these experiments great care must be taken not to carry over with the inoculant an appreciable quantity of —SH.

A hypothesis which will satisfactorily interpret the action of oxygen must account for the following facts:

(1) that a fresh culture of *B. sporogenes* inoculated into TB exhibits a relatively small latent period in its anaerobic growth;

(2) that a culture of the organism, oxygenated for some time, exhibits when cultivated under strictly anaerobic conditions, a long latent period in TB but not in TB—CSH;

(3) that this long latent period is not due to the presence of oxygen or peroxides in the inoculant.

We have suggested that this latent period may simply be the time of incubation necessary for the inoculated non-proliferating cells of *B. sporogenes* to produce, by proteolytic activity, a minimal quantity of —SH. As soon as this amount is formed proliferation commences. The results with gelatin support this suggestion.

A ready explanation for the behaviour of oxygen is now forthcoming; for if the —SH formed by the organism from TB is removed by oxygen at a rate at least equal to the velocity of its formation then the minimal concentration of —SH is never produced and proliferation cannot occur. If —SH be initially present (as in TB—CSH) then not only will the —SH protect the cell from oxygen, for a time dependent on its concentration, but it will secure conditions for immediate proliferation of the inoculated organism. Such proliferation will result, by proteolytic activity, in the production of more —SH. Thus, even if the initial quantity of —SH in the medium were such as to require relatively little time for its oxidation, yet in this time proliferation may have commenced with the consequent production and maintenance of a steady supply of —SH from the medium. The problem, indeed, resolves itself into one of relative velocities; if the velocity of oxidation of the —SH be greater than the rate of its formation, then proliferation cannot occur; if proliferation has already commenced by reason of the initial presence of a quantity of —SH then proliferation will continue as long as the minimal concentration of —SH is maintained.

We do not, at present, however, consider that only the —SH group has this effect in securing the condition necessary for the proliferation of *B. sporo-*

genes. Our experience points to the fact that glucose also may have an effect similar to —SH, but the effect seems, in the case of *B. sporogenes*, initially, to be much less powerful.

A most important fact concerning the —SH group (as illustrated by cysteine and glutathione) is its ability to give a reduction potential which is not influenced by the presence of the oxidised (—S—S—) form. This, it appears to us, is the significant fact underlying the phenomena we have described. Even very low concentrations of cysteine, as an examination of the relationship between reduction potential and concentration of this substance [Dixon and Quastel, 1923] will indicate, give high reduction potentials; solutions so dilute that the nitroprusside reaction is indefinite give relatively high potentials. In view of this fact we may restate the hypothesis put forward earlier.

Let us assume that before proliferation of *B. sporogenes*—or of any cell which leads a "strictly" anaerobic career—can occur, the cell must have attained a certain limiting reduction potential. At any potential more oxidising than this the cell fails to proliferate. At or below this potential proliferation is possible. The potential may be secured by the presence of a small quantity of an —SH compound or it may obtain in the presence of glucose or a variety of substances characterised by their reducing behaviour in presence of the cell.

The effect of oxygen in preventing the proliferation of strict anaerobes would then be due to its removal of substances responsible for the maintenance of the critical reduction potential with the consequent raising of the potential above the limiting point.

A fresh culture of *B. sporogenes* is at or below its limiting reduction potential and hence its inoculation into a medium[1] which will not markedly affect this potential will be followed by a relatively rapid proliferation. Its inoculation, however, into one which increases the potential above the limiting point will result in failure to proliferate, until the organism has, itself, by its proteolytic (or other) activity, induced the formation of substances creating the requisite limiting potential. Similarly, a culture of *B. sporogenes* which has been oxygenated, or treated in some such way, will possess a potential above its critical point and its inoculation into an inert (*i.e.* from the point of view of affecting the potential) nutritional medium will result in the manifestation of a long latent period in its anaerobic growth. On subculture, however, into a medium already possessing a high reduction potential (*e.g.* TB—CSH) this latent period will be diminished or removed. The latent period, it is clear, is a function of (1) the nature of the medium or environment, (2) the power of the cell to produce substances creating a reduction potential, (3) the value of the cell's characteristic limiting reduction potential.

Potentiometric evidence, such as that obtained by Cannan, Cohen and

[1] It is assumed of course that the medium possesses the nutritional requirements of the organism.

Clark [1926] with certain organisms, would, probably, be of considerable value in this connection.

We may now consider the effects of exposing an anaerobe such as *B. sporogenes* to hydrogen peroxide. There is little doubt that in a growing culture of *B. sporogenes* containing —SH compounds and exposed to the air, there is formation of hydrogen peroxide. McLeod and Gordon's observations [1925, 1, 2], taken together with the recent work on oxidations in presence of cysteine or glutathione, make this highly probable. We have seen, however, that in spite of such a production of peroxide the organism is not killed. McLeod and Gordon have shown that anaerobes do not proliferate in media containing extremely small quantities of hydrogen peroxide (at concentrations of the order of two or three parts in a million) and have adduced this observation as evidence for the toxic nature of peroxide.

It is clear, on our views, that the effect of such small concentrations of peroxide will be similar to that of oxygen itself. It follows that, if this hydrogen peroxide be removed, after exposing the organism to it, and the correct reduction potential be set up, proliferation of the organism should occur. Nevertheless, we must expect that at some relatively high concentration, hydrogen peroxide will be actually toxic, *i.e.* its action will be irreversible, for at this concentration it will attack the substance of the organism to such an extent that death must ensue. The addition of any reducing substances can, then, have no reviving influence. Hence we may expect hydrogen peroxide to exhibit two critical concentrations—a lower one whose effect is reversible and which, acting like oxygen, increases the oxidation potential of the cell above the limiting point and thus prevents proliferation; and a higher one whose effect is irreversible and by directly oxidising the substance of the organism produces an irreversible condition; this may be regarded as the toxic or lethal concentration. Thus, between the two critical concentrations, we should be able to observe effects precisely similar to those obtained with oxygen and which only differ in their quantitative aspect. These expectations are fully borne out by experiment.

Exp. 8. A series of 11 tubes, *A*, *B*, ... *K*, was prepared so as to contain 10 cc. each of tryptic broth with concentrations of H_2O_2 varying from 0·03 % to 0·003 %. This was done by placing the appropriate volume of broth (9 cc., 9·1 cc....9·9 cc.) in each tube, autoclaving the tubes and then adding the necessary volume of 0·3 % H_2O_2 (1 cc., 0·9 cc....0·1 cc.) by means of a sterile pipette. These were prepared in duplicate so that finally there were two series of tubes containing diminishing quantities of H_2O_2, the highest concentration being 0·03 % in *A*, that in *J* being 0·003 % and that in *K* being zero. Each tube was then inoculated with three drops of a fresh culture of *B. sporogenes* in TB and incubated anaerobically at 37°. After 18 hours no growth was observed in any tube except in the two *K*'s where growth was abundant. So far McLeod and Gordon's observation of the growth inhibiting effect of very small quantities of hydrogen peroxide on the anaerobe is

confirmed, *i.e.* a concentration of 0·003 % H_2O_2 appears to be toxic. One series of the tubes was then marked A', B', ... J' and to each tube was added 1 cc. of neutralised sterile 10 % solution of cysteine hydrochloride. This amount of cysteine was sufficient to react with the highest concentration of H_2O_2 present in the tubes and to leave sufficient —SH over to give a strong nitroprusside reaction. The other series, A, B, ... J, was not disturbed. Both series were incubated anaerobically for a further 48 hours. The results are shown in Table IV.

Table IV.

Medium without added CSH ...	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*
Initial concentration of H_2O_2	·03 %	·027	·024	·021	·018	·015	·012	·009	·006	·003
Growth after further 48 hours ...	0	0	0	0	0	0	0	+ +	+ + +	+ + +
Medium with added CSH	*A′*	*B′*	*C′*	*D′*	*E′*	*F′*	*G′*	*H′*	*I′*	*J′*
Growth after further 48 hours ...	0	0	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +

The experiment shows (1) that the presence of very small quantities of H_2O_2 has an inhibiting influence on the growth of *B. sporogenes*; (2) that this inhibiting influence is similar to the effect of oxygen, for, on further incubation, growth occurs up to a tube which contained initially 0·009 % H_2O_2; (3) that the subsequent addition of cysteine makes growth possible in a tube which contained initially 0·024 % H_2O_2.

It follows that a concentration of H_2O_2 as high as 0·024 % is not lethal to all the organisms. Usually we have found no growth in A' or B' so that it would appear that the lethal concentration of H_2O_2 lies between 0·027 % and 0·024 %. On one occasion, however, we found a slight growth of the anaerobe in A'. The fact that growth occurs in J, I, H, where cysteine was not added, is due, doubtless, to the removal of the added H_2O_2 by the constituents of the broth, the organism then proceeding to create its limiting reduction potential in the manner we have described. The effect of cysteine is simply to accelerate the process of removal of H_2O_2 to a very marked extent and to create, itself, very quickly the necessary reduction potential for proliferation.

General Considerations.

The views we have put forward seem to us to provide a satisfactory interpretation not only of the results obtained by McLeod and Gordon [1925, 1, 2] in their experiments on chocolate agar, but of Callow's, of Novy's and of a variety of other observations on the growth of the strict anaerobes.

It is well known, for instance, that a large inoculation of *B. sporogenes* into glucose broth (and sometimes plain broth) will almost certainly give a good aerobic growth, whereas a small inoculation will give indecisive and

often negative results. It is easy to see why this must be, for with a large inoculation not only a large number of cells are introduced but an appreciable quantity of —SH from the inoculating fluid. This secures the necessary reduction potential for proliferation even in the presence of oxygen. If the quantity of —SH inoculated be small, the —SH may be oxidised before proliferation has commenced and hence the failure to grow. As stated earlier, the question becomes one of relative velocities; if once the organism can begin to proliferate and produce a stream of —SH compounds, or other substances of similar reducing nature, at a velocity greater than that at which they are removed by oxygen, aerobic growth will be possible. Catalase fails to secure aerobic growth simply because it cannot induce the correct reducing potential; its presence, however, protects the organism from the truly toxic irreversibly acting concentration of H_2O_2.

Since the velocity of oxidation is determined partly by the tension of oxygen it follows that different anaerobes will proliferate at different critical tensions of oxygen, these tensions being determined by the relative velocities of formation of substances responsible for the maintenance of the reduction potential and of their oxidation by oxygen. Similarly, different anaerobes will exhibit different susceptibilities to exposure to oxygen or hydrogen peroxide, the latent period in anaerobic growth being determined by the medium, the proteolytic activity (or power to produce reducing substances) and the value of the limiting reduction potential. We have confined our attention almost exclusively to *B. sporogenes*—a strongly proteolytic organism. With the highly saccharolytic and less proteolytic organisms it may be that —SH does not play so predominant a part and that the powerful activation of glucose, with consequent high reducing power, becomes the important factor.

When a substance does not give a reduction potential observable at the electrode it depends for its reducing power *in vivo* on its activation by a cell, or enzyme. Now formates are very highly activated by an organism such as *B. coli* and would, undoubtedly, have high reducing power in presence of this organism. It must not be concluded, however, that formates are "reducing" in presence of *any* organism. Our own observations indicate that formates are but feebly activated by *B. sporogenes* and in correlation with this we find that formates have very little or no effect compared with —SH.

An interesting point in connection with —SH is the effect of fresh tissues on the growth of the anaerobes. It is well known that such tissues are able to induce prolific growth, even aerobically, of most anaerobes. Now the effect may be due in part to respiration of the tissue, as Novy has pointed out, and it may be due, in part, to the presence of certain nutritionally valuable substances, but it is significant that with the animal tissues the latter not only contain a supply of —SH (in the form of glutathione) but a mechanism by which a certain supply can be maintained even aerobically [Hopkins, 1921; Hopkins and Dixon, 1922]. By this last circumstance alone an organism

such as *B. sporogenes* will find in an animal tissue its ideal *milieu*. Even the presence of autoclaved animal tissue will be extremely valuable in a medium which initially contains a little —SH or —S—S—, for the tissue still retains its power of producing —SH from —S—S— and hence of maintaining the supply of —SH.

Viability.

We have carried out many experiments to determine whether the presence of 0·1 % cysteine hydrochloride in a medium affects the viability of *B. sporogenes* and so far we have received, by repeated subculture into a medium containing 0·1 % cysteine hydrochloride, no indication that the substance has any harmful effect whatever on the organism.

Summary.

1. Neither oxygen nor hydrogen peroxide up to a concentration of 0·024 % is lethal to *B. sporogenes*.

2. *B. sporogenes*, after it has been exposed to oxygen or dilute concentrations of hydrogen peroxide, exhibits a long latent period in its anaerobic growth in tryptic broth.

3. This latent period is diminished or almost entirely removed by the presence of cysteine (or other —SH compound) in the medium.

4. The presence of a small amount of cysteine (or other —SH compound) in tryptic broth will induce good aerobic growth of *B. sporogenes*.

5. It is suggested that the latent period exhibited by oxygenated *B. sporogenes* is the time of incubation required for the inoculated non-proliferating cells of the organism to produce a certain minimum quantity of —SH (or other reducing bodies) from the medium.

6. This minimum quantity of —SH (or other reducing bodies) must be present in order to establish the limiting reduction potential which is necessary for proliferation of the organism. In a medium which maintains a higher (more oxidising) potential than this, proliferation of the anaerobe cannot occur. In a medium in which the cell is at or below this potential proliferation is possible.

7. It is shown that, on the assumption of the existence of a limiting reduction potential, which is necessary for the proliferation of an "obligate" anaerobe, all observations on the relationships of the anaerobes to oxygen can be satisfactorily interpreted.

It is a great pleasure to express our appreciation of the interest taken by Sir F. G. Hopkins in this work. One of us (M. S.) is in receipt of a grant from the Medical Research Council.

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NOTE. (*Added November 1st*, 1926.) Whilst this communication was in the press the work of Hosoya, and Hosoya and Kishino (Scientific Reports from the Government Institute for Infectious Diseases. Tokyo, 1925, pp. 103 and 123) came to our notice. The Japanese investigators have established the following facts: that broth containing 0·001 % cysteine hydrochloride admits of rapid growth of various anaerobic bacilli and that —SH will induce the growth of strict anaerobes on a digest of gelatin which in the absence of the —SH is unsuitable for such growth. They have not, however, attacked the problem of the relationship of the anaerobes to oxygen in the manner developed in this paper; nor have they arrived at our conclusions.

CXLIV. OXIDISING ENZYMES.

IX. ON THE MECHANISM OF PLANT OXIDASES.

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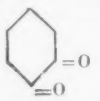
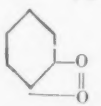
On a basis of the original conception of Bach and Chodat, the authors have considered the mechanism of the oxidase of the higher plants to be as follows: first, the formation of an organic peroxide from the dihydroxy-group characteristic of catechol, this reaction being catalysed by an oxygenase; second, the decomposition of this peroxide (or of hydrogen peroxide derived from it by the action of water) with the production of active oxygen, this reaction being catalysed by a peroxidase. The active oxygen so formed will oxidise guaiacum and other acceptors. The actual form of peroxide derived from the dihydroxy-group has not been postulated.

Recently Szent-Györgyi [1925] has made the enlightening suggestion, supported, to some extent, by experimental evidence, that an orthoquinone is the product of the action of the oxygenase upon catechol. This orthoquinone, moreover, will blue guaiacum in the absence of peroxidase. Hence, Szent-Györgyi has concluded that peroxidase is not a component of the type of oxidase under consideration. Orthoquinones, of two types:

(i)   (ii)

have been prepared by the oxidation of catechol with silver oxide by Willstätter and Müller [1908]. Owing to its far greater stability, Szent-Györgyi has suggested type (i) as the probable product formed by the oxygenase. Such a product prepared artificially will, he has shown, blue guaiacum.

In addition to the above modification of the conception of the oxidase mechanism, Szent-Györgyi is also of the opinion that peroxidases are merely attenuated oxidases and do not exist as specific enzymes. The absence of oxidases from, and the presence of authenticated peroxidases in, a certain percentage of the higher plants is due, in his opinion, to the absence of "catechol" compounds, the place of which can be taken by hydrogen peroxide. With this point of view we do not agree. Not only do the non-oxidase plants

contain no catechol compounds, but they contain no oxygenase which is capable of acting upon the catechol group, even when supplied.

The experimental evidence, offered by Szent-Györgyi in support of the view that the blueing of guaiacum in the oxidase reaction is due solely to orthoquinone, is as follows. An enzyme preparation, from the potato tuber, was allowed to act upon catechol in potassium dihydrogen phosphate solution. After a certain interval, excess of methyl alcohol was added for the precipitation of enzymes. The oxidised product (presumed to be orthoquinone), however, remained in solution, and, after the careful removal of alcohol by evaporation at low temperature and reduced pressure, caused the blueing of guaiacum. The above is no evidence, of course, for the formation of an orthoquinone, but demonstrates only that an oxidation product has been produced which will blue guaiacum in the absence of any enzyme.

Szent-Györgyi has stated that two factors have a considerable influence on the progress of the reaction, the concentration of catechol and the hydrogen ion concentration. From experimental evidence he has shown the optimum concentration of catechol to be about 0·005 %. The intensity of the guaiacum reaction decreased with higher concentrations of catechol. This, he has suggested, may be due to a reaction between the quinone and the unchanged catechol to form less active compounds.

With regard to hydrogen ion concentration, he found that at p_H 7·4 the oxidation of catechol was relatively rapid, but the guaiacum blue tended to fade. With increasing acidity, the oxidation of catechol takes place more slowly, but the blue colour is more stable. This, he has suggested, may be explained on the grounds that the above-mentioned combination of oxidised and unaltered catechol takes place more readily with increasing alkalinity. For a 10-minutes' period of reaction between enzyme and catechol, the optimum p_H appeared to be 6·4, which he also found to be that of potato juice.

The authors have repeated, exactly, as far as they can judge, the experimental procedure of Szent-Györgyi. In addition, a control experiment was performed using enzyme without catechol. In the latter, after removal of enzyme and alcohol, a test for peroxidase was carried out with hydrogen peroxide and guaiacum. Whereas the actual experiments were positive, it was found that this so-called control, as often as not, gave a peroxidase reaction; that is, the conditions of the experiment did not necessarily ensure complete precipitation of the peroxidase. Hence it formed no control, nor can one be sure, when a blue colour was given in the actual experiment with catechol, that it was not due to traces of peroxidase unprecipitated by alcohol.

In the above experiments a 0·2 *M* solution of potassium dihydrogen phosphate (p_H 4·6) was always employed. Subsequently, the concentration of alcohol was increased four times without necessarily giving a negative result in the so-called control.

Hence, by means of Szent-Györgyi's technique as employed by us, we were unable to ensure the absence of peroxidase.

A series of experiments was next performed identical with the above, except that distilled water was employed instead of phosphate solution. The results were, with one doubtful exception, negative; namely, no blueing of guaiacum was obtained. The control experiments, after addition of hydrogen peroxide, were almost always positive. From the above results, the conclusion may be drawn that, at approximately p_H 7, the "orthoquinone" is not stable, but is removed by condensation or otherwise. The positive results with the control may possibly be explained by the fact that in water the enzymes were less completely precipitated by alcohol than in phosphate solution.

A modification of the experiment was made by adding an extract of horseradish peroxidase to a solution of the "orthoquinone" after removal of alcohol. On subsequent addition of guaiacum, no intensification of blueing was observed, as compared with a control without peroxidase. From this crude experiment, it does not appear that peroxidase catalyses the oxidation of guaiacum by the "orthoquinone."

On the assumption that the alcohol technique may not be valid, we tried to solve the problem by substituting other enzyme precipitants in Szent-Györgyi's method. Those selected were colloidal ferric hydroxide, barium chloride, tannic acid, mercuric chloride and Esbach's reagent (1 g. picric acid and 1 g. citric acid per 100 cc. of water).

Colloidal ferric hydroxide. Three times the quantities given by Szent-Györgyi were used, namely, 90 mg. of enzyme, 3 cc. of phosphate and 3 drops of 0·1 % catechol solution. These were incubated at 37°, 1·5 cc. of colloidal ferric hydroxide was added, the solution filtered from the precipitate, and tested with guaiacum. The results were always positive, the controls without catechol negative. Though, naturally, the use of colloidal ferric hydroxide is open to criticism in connection with guaiacum, not only did the ferric hydroxide precipitates fail to give any positive reaction with this reagent, but control experiments with catechol and phosphate, incubated and subsequently precipitated with colloidal ferric hydroxide, were also negative.

Barium chloride and mercuric chloride. The precipitation of the enzyme was not complete. Of other mercury salts, mercuric nitrate itself was found to blue guaiacum.

Tannic acid. This reagent completely inhibited the blueing of guaiacum by the "orthoquinone."

Esbach's reagent. This was successful as in the case of colloidal ferric hydroxide. For 90 mg. of enzyme, etc., 0·5 cc. of the reagent was used. In spite of the colour of the picric acid, the blueing of guaiacum could be distinctly seen.

Colloidal ferric hydroxide was also employed with an aqueous catechol solution without phosphate. It was, however, unsuccessful. If sufficient iron were used to ensure complete precipitation of the enzyme, the ferric hydroxide itself was not precipitated. The use of Esbach's reagent with aqueous catechol

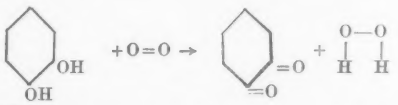
was successful in removing the enzyme, and the results were negative as determined by the guaiacum reaction.

From the above, we conclude that, within a certain range of hydrogen ion concentration, an oxidation product (possibly orthoquinone) is formed from catechol as a result of catalysis by oxygenase, and this product is capable of blueing guaiacum in the absence of any enzyme.

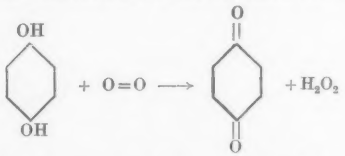
In a previous communication [Onslow and Robinson, 1925], the authors have shown that when a potato enzyme preparation is allowed to act on *p*-cresol, a dihydroxy-compound, presumably 3·4-dihydroxytoluene, is first formed, and is subsequently oxidised by the oxygenase. This statement is also supported by the observations of Uys-Smith [1926], and if correct, it should be possible to detect the formation of an "orthoquinone," as it is in the case of catechol.

Experiments were therefore made using *p*-cresol, instead of catechol, in phosphate solution with colloidal ferric hydroxide. A positive reaction was obtained with guaiacum, showing that an analogous oxidation product has been formed from *p*-cresol.

A further aspect of the problem will now be considered. If the view be accepted that orthoquinone is the probable oxidation product of catechol in the oxidase system, then a simple expression of the reaction would be as follows:



In connection with this question of the nature of the products formed on oxidation of catechol, some recent work of Wieland and Fischer [1926] is of importance. In these researches, the applicability of Wieland's conception of oxidation, by means of hydrogen activation and transport to molecular oxygen, has been tested in the case of oxidation of various phenols by a Basidiomycete catalyst, namely that of *Lactarius vellereus*. From this fungus, preparations were made which contained a system (whether equivalent to an oxygenase we cannot say) which was eventually obtained almost entirely free from peroxidase and catalase by fractional precipitation. The action of such a system on quinol was accompanied by a production of hydrogen peroxide in amount approaching the theoretical value required for the reaction:



Hydrogen peroxide was also obtained in considerable quantity from the action of the system upon catechol, pyrogallol and guaiacol. Wieland and

Fischer were, however, unable to obtain dehydrogenation anaerobically when molecular oxygen was replaced by methylene blue and various other hydrogen acceptors.

To determine whether, in the higher plants, hydrogen peroxide is formed simultaneously with the quinone is a difficult problem. If we assume the above equation to be a correct representation, then in a mixture of an enzyme (potato) preparation and an aqueous solution of catechol, the following are present: oxygenase, peroxidase, catalase (probably) and catechol: orthoquinone and hydrogen peroxide are produced. The former tends to disappear, we may suppose, by condensation. It would seem, moreover, that under these conditions, if any hydrogen peroxide were formed, it would tend to be decomposed either by (*a*) peroxidase giving active oxygen which can be accepted by unchanged catechol, or (*b*) catalase, giving molecular oxygen.

On addition of guaiacum to such a system, it would be oxidised by the orthoquinone. It might also be oxidised by the hydrogen-peroxide-peroxidase system, though we do not know how the hydrogen peroxide may be distributed in the reactions in which catalase, peroxidase, guaiacum and catechol are involved.

Nevertheless, an attempt was made to see if it were possible to detect the presence of hydrogen peroxide from the oxidation of catechol. When an aqueous solution of catechol is left in air, it slowly oxidises and becomes brown. Such an autoxidised solution will not blue guaiacum alone, but will do so on addition of peroxidase (from horse-radish, etc.). Hence we must conclude that a peroxide is present. Titanium sulphate appears to be the only reagent which discriminates between organic peroxides and hydrogen peroxide. We therefore proceeded as follows: a 5 % aqueous solution of catechol was kept at 37° for 12 hours. Since we found that catechol itself gives a yellow colour with titanium sulphate, the autoxidised solution was extracted with specially purified ether until all catechol was removed (as detected by the ferric chloride reaction). After removal of the catechol, titanium sulphate solution was added. A definite yellow colour appeared. As a control we tested a freshly prepared catechol solution which had been extracted with the same sample of ether. This form of control we found especially necessary, since the washings from ordinary laboratory ether were found to give very strong reactions with titanium sulphate owing to the presence of peroxide. Hence, for the above extraction of catechol, we employed purified ether which had been washed with water and distilled from concentrated sulphuric acid and afterwards from sodium. As an additional confirmation of the above test with titanium and the autoxidised catechol, it was repeated with the following modification: the solution, after ether extraction, was divided into two portions, and to one-half we added liver catalase which had been freed from peroxidase by filtration through a Chamberland candle. On the addition of titanium sulphate no yellow colour appeared in the half to which catalase had been added, though the control gave a positive reaction. From the above experiments

we assume that, in the autoxidation of catechol, hydrogen peroxide is most probably formed.

The detection of hydrogen peroxide when the oxidation is catalysed by an oxygenase was also attempted. The potato enzyme was allowed to act on a 2·5 % solution of catechol in distilled water at 37° for about 30 minutes. The mixture was then extracted with peroxide-free ether until free from catechol. A distinctly positive reaction was given with titanium sulphate; this reaction, moreover, did not appear after the addition of catalase. As far as these qualitative tests are valid, we draw the conclusion that hydrogen peroxide is probably formed when the oxidation of catechol is catalysed by the oxygenase. It is apparent, however, that the difficulty of demonstrating the presence or absence of hydrogen peroxide in the higher plants may be largely due to the fact that a separation of oxygenase from peroxidase and catalase has not yet been achieved.

The facts so far described lead to the possibility of considering the oxygenase of the higher plants as a dehydrogenase. In this case it is of some importance to find out whether catechol can be oxidised anaerobically, molecular oxygen being replaced by other hydrogen acceptors.

Attempts to oxidise catechol anaerobically in the presence of oxygenase, using methylene blue as a hydrogen acceptor, have so far been unsuccessful. In view of this result, advantage was taken of a new series of indicators introduced by Mansfield Clark [Needham and Needham, 1926]. We are indebted to Dr J. Needham for samples of these, and to Dr M. Dixon for assistance in making up the necessary solutions.

The indicators used were *o*-cresol-2·6-dichloroindophenol, *o*-cresolindophenol, 2·6-dibromophenolindophenol and *o*-chlorophenolindophenol. All are reduced more readily than methylene blue and are arranged above in order of increasing reducibility.

The experiments were conducted in Thunberg tubes carefully evacuated and tested during the course of the experiments, the tubes being kept in a bath at 37°.

The enzyme extracts were made by a method, previously described [Onslow, 1920], of extracting the residue (prepared by treating the tissues with alcohol) with water and filtering; or, in some cases, by precipitation of the water extract with alcohol and again taking up in water. For control experiments, the enzyme solution was well boiled, filtered and well cooled in air. Phosphate buffers were employed ranging from p_H 5·8–7·0. The concentration of catechol solution was 0·1 %.

Definite amounts of buffer, enzyme extract and indicator were introduced into the Thunberg tube. The catechol solution was always measured into a very small test-tube placed within the Thunberg tube. By this means any oxidation, before complete evacuation, was avoided. After evacuation, the liquids were mixed by inverting the tube. A control, using boiled enzyme, was always set up, and, in some cases, controls of enzyme and indicator alone,

and catechol and indicator alone. The time for complete decoloration was noted.

The above indicators were reduced by catechol alone, given a sufficient period of time. The object of the research was to ascertain whether, in plants containing the oxygenase-catechol system, the enzyme catalysed the reduction of these indicators. If so, the inference may be drawn that catechol is simultaneously oxidised as a result of hydrogen transference.

A number of oxidase and non-oxidase plants were investigated as follows:

	Time in minutes for decoloration		
	Unboiled	Boiled	Control
Oxidase plants			
1. Potato tuber	9	24	180
2. Artichoke tuber	25	45	—
3. „ leaf	10	30	195
4. Banana fruit	Boiled and unboiled almost simultaneously in 40 minutes		180
5. Lilac leaf	Both in 87 minutes		—
6. Apple fruit	Unboiled, slightly paler in 120 minutes		—
Peroxidase plants			
7. Horse-radish root ...	60	30	—
8. „ ...	Both in 30 minutes		—
9. Chickweed (*Stellaria*) plant	Both simultaneously		—
10. Cucumber fruit	Both in 230 minutes		—
11. „	100	130	—
12. Turnip root	70	90	—
13. „	70	100	—
14. „	55	100	—
15. „	60	80	—

In the case of the potato tuber, artichoke tuber and turnip root, both crude extract and solution of precipitated enzyme were used.

It should be noted that when several experiments were performed, different enzyme preparations were used. Owing to the impossibility of obtaining comparable strengths of enzyme when using crude extracts, concordance in the times of decoloration for separate experiments cannot be expected. When enzyme preparations were made from leaves, difficulty was experienced in extracting the enzymes from the tissue residue, especially when tannins are present, as is usually the case. These appear to render the enzymes precipitated *in situ* much less soluble.

In addition to the above, preparations were made from leaves of dead-nettle (*Lamium*) and goutweed (*Aegopodium*), both oxidase plants, and from iris rhizome and dried peas, both tissues of non-oxidase plants. In these cases the enzyme extracts reduced the indicators rapidly in air.

From the above data, we conclude there is very little, if any, evidence for an enzymic reduction of the indicators, the decrease in rate in the case of the boiled enzyme being possibly due to removal of proteins[1]. Since the results of using enzymes from both oxidase and peroxidase plants are, on the whole, similar, there is no evidence from these experiments for the point of view

[1] Pure pseudoglobulin, kindly prepared for us by Mr G. S. Adair, decolorised the indicator in 2 hours, either in the presence or absence of catechol, whereas boiled controls did not.

that the oxygenase, acting as a dehydrogenase, can bring about an anaerobic oxidation of catechol.

SUMMARY.

1. We have confirmed the observations of Szent-Györgyi, that an oxidation product, formed by the action of the oxygenase of the potato tuber on catechol, is capable of blueing guaiacum in the absence of peroxidase.

2. The procedure adopted by Szent-Györgyi for removal of peroxidase by precipitation with methyl alcohol was not successful when applied by us. When colloidal ferric hydroxide or picric acid was substituted for alcohol, the removal of peroxidase appeared to be complete.

3. The suggestion offered by Szent-Györgyi, that the above-mentioned oxidation product may be orthoquinone, is favourable to the hypothesis of oxidation of catechol, by dehydrogenation, with the formation of hydrogen peroxide with molecular oxygen. The oxygenase in this case would be a dehydrogenase.

4. As far as could be detected qualitatively with titanium sulphate, hydrogen peroxide appears to be formed during the autoxidation of catechol: it is probably also produced when the oxidation is catalysed by oxygenase.

5. Experiments were carried out to ascertain whether the oxygenase, assuming it to be a dehydrogenase, is capable of oxidising catechol anaerobically in the presence of a hydrogen acceptor. Methylene blue and the other more readily reducible indicators of Mansfield Clark were employed. No evidence was obtained from these experiments that oxygenase can act in this capacity.

In conclusion we are much indebted to Sir F. G. Hopkins for many suggestions and criticisms.

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